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Overwintering biology of the brown marmorated stink bug, Halyomorpha halys (Hemiptera: Pentatomidae)

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Abstract

The invasive crop pest *Halyomorpha halys* has been established in Ontario since 2012 and poses a significant threat to Canadian agriculture. I investigated the tolerance of *H. halys* to three stressors - low temperatures, desiccation, and energy depletion - in addition to investigating the role of lab-induced diapause in enhancing stress tolerance. Overwintering *H. halys* depress their supercooling point to -15.4 °C and LT_{50} to -17.5 °C after acute (1 h) exposure, however they do not encounter these temperatures while overwintering indoors. Moreover, overwintering *H. halys* maintain their water balance through a reduction of water loss rates, while conserving energy stores (lipids and carbohydrates). This is consistent with lab-reared-diapausing *H. halys* who exhibit reduced water loss rates and temperature-independent metabolic suppression relative to non-diapausing adults, suggesting that diapause enhances desiccation resistance and energy conservation. Thus, *H. halys* are likely to persist in Ontario barring any significant changes in overwintering conditions.

Keywords

Halyomorpha halys, overwintering, diapause, stressor, cold tolerance, desiccation, energetics, seasonal plasticity

Co-Authorship Statement

This work was conducted under the supervision of Dr. Brent J. Sinclair and Dr. Tara D. Gariepy. All aspects of sampling, experimental design, and analysis were planned in coordination with Drs. Sinclair and Gariepy, and publications resulting from this work will be co-authored with them.

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1 Introduction

Overwintering insects encounter a variety of stressors – including low temperatures, desiccation, and energy depletion – which may cause mortality and dictate a species' overwintering success (Sinclair et al., 2013; Sinclair, 2015). Invasive species establish in areas outside of their native range, and the extent to which they can further expand their range is dependent on the climate and environmental conditions of their invaded habitats. Understanding how an invasive species responds to overwintering stressors is therefore imperative for predicting their establishment and future range expansion. *Halyomorpha halys*, the brown marmorated stink bug (Hemiptera: Pentatomidae) is native to Southeast Asia and has been established in Ontario since 2012 (Gariepy et al., 2014a). *Halyomorpha halys* feeding causes severe economic damage to a wide variety of fruit and vegetable crops, and given its wide host range (Philips et al., 2016) *H. halys* poses a significant threat to the Canadian agricultural landscape. Preliminary studies have investigated some aspects of *H. halys* cold tolerance (Cira et al., 2016; Lowenstein and Walton, 2018), however the information available does not describe the full extent of their cold-hardiness. Moreover, a gap in knowledge remains regarding the ability of *H. halys* to tolerate additional stressors while overwintering. Thus, I assessed the tolerance of *H. halys* to low temperatures, desiccation, and energy depletion in Ontario, and investigated the role of diapause in enhancing stress tolerance.

1.1 Insects at low temperatures

As ectotherms, the performance and survival of insects is influenced by shifts in temperature and associated abiotic stressors (Sinclair et al., 2015). In temperate North America, insects spend most of their life overwintering and have developed several key life history adaptations that promote overwintering success under unfavourable environmental conditions. The ability to tolerate environmental stress is therefore important in determining a species' overwintering success (Williams et al., 2015b).

Insect body temperature changes with environmental temperature, and at extreme low temperatures (i.e. subzero), many insects risk mortality. As such, low temperatures not only determine survival but also population growth and subsequent range distribution (Addo-Bediako

et al., 2000; Boggs and Inouye, 2012; Halbritter et al., 2018). ; Species living at higher latitudes are likely to experience lower temperatures compared to those closer to the equator, and often express enhanced tolerance to low temperatures (Addo-Bediako et al., 2000). This relationship is important when determining invasive pest establishment; range expansion of an invasive species is limited by its ability to tolerate adverse environmental conditions, including low temperatures (Addo-Bediako et al., 2000). For example, the invasive emerald ash borer, *Agrilus planipennis* (Coleoptera: Bupresitdae), was able to establish in Canada due to its cold tolerance (Crosthwaite et al., 2011; Cuddington et al., 2018). Conversely, spotted wing drosophila, *Drosophila suzukii* (Diptera: Drosophilidae), is probably geographically limited because of a lack of cold tolerance (Jakobs et al., 2015), as is the Mexican pine white, *Neophasia terlooii* (Lepidoptera: Pieridae), whose geographic range is limited by cold winter temperatures in northern Arizona (Halbritter et al., 2018).

1.1.1 Cold tolerance strategies

The ability to survive low (sub-zero) temperatures is variable in insects, who are categorized as either chill-susceptible, freeze-avoidant, or freeze-tolerant depending on their capacity to survive internal ice formation (Table 1.1; Sinclair et al., 2015). Freeze-avoidant species die at the temperature at which internal ice formation begins - the supercooling point (SCP) - while freeze-tolerant species are capable of surviving freezing (Sinclair et al., 2015). Chill-susceptible insects are the least cold-hardy and die from chilling-related injuries at temperatures above their SCP (Lee, 2010). The SCP can be used as a metric of cold tolerance in freeze-avoidant species, given that freezing is lethal (Sinclair et al., 2015). However, because chill-susceptible and freeze-tolerant insects die at temperatures above and below their SCP, lower lethal temperature (LLT) – the temperature at which mortality occurs after acute exposure (e.g. < 6 h) to low temperatures – is the most appropriate metric of their cold-hardiness species (Sinclair et al., 2015). Additionally, exposure time is important for interpreting a chill-susceptible or freeze-tolerant insects' cold-hardiness; many investigations hold insects at constant temperatures for an extended period (e.g. > 1 day) to provide an estimate of lethal time (Lt) to mortality (Sømme, 1996; Turnock and Fields, 2005).

Table 1.1. Cold tolerance strategies (Lee, 2010) as used by *Eurosta solidaginis* (Irwin and Lee, 2003), *Agrilus planipennis* (Crosthwaite et al., 2011), and *Drosophila suzukii* (Jakobs et al., 2015). Asterisk indicates that chill-susceptibility is not a true cold tolerance strategy, but rather a description of the response to cold exposure.

Cold Tolerance Strategy	Definition	Example Species
Freeze-Tolerance	Species which tolerate and survive internal ice formation	*Eurosta solidaginis*
Freeze-Avoidance	Species which depress their supercooling point, but die once bodily fluids begin to freeze	*Agrilus planipennis*
***Chill-Susceptibility**	Species which die at temperatures above their supercooling point	*Drosophila suzukii*

Insects have several different mechanisms which enhance survival at low temperatures: for example freeze-tolerant species, such as the woolly bear caterpillar, *Pyrrharctia isabella* (Lepidoptera: Erebidae), survive freezing by producing ice-nucleators that promote controlled extracellular ice formation (Lee, 2010; Duman et al., 2010), while freeze-avoidant species such as *A. planipennis* depress their SCP through the production and accumulation of low-molecular-weight hemolymph cryoprotectants, but die upon freezing (Crosthwaite et al., 2011; Sinclair et al., 2015). Moreover, many freeze-tolerant and freeze-avoidant species produce anti-freeze proteins which bind to the surface of ice crystals and prevent recrystallization Zachariassen, 1985; Duman et al., 2010). Most insect species are chill-susceptible, however, and die from chilling-related injuries. Chill-susceptible insects enter chill coma – a reversible state of paralysis – at their critical thermal minimum (CT_{min}), the temperature at which ion and water homeostasis are lost, and muscle function stops (MacMillan and Sinclair, 2011a; Des Marteaux et al., 2018). If the cold exposure is intense or prolonged, chilling injuries will accumulate; in the Fall field cricket, *Gryllus pennsylvanicus* (Orthoptera: Gryllidae), for example, exposure to 0 °C causes chilling injury in as little as 12 hours, and mortality after 3-5 days (MacMillan and Sinclair, 2011b; Des Marteaux et al. 2018). To recover from cold exposure, chill-susceptible species must therefore re-establish ion and water balance and repair chilling injuries ; (MacMillan et al., 2012; Overgaard and MacMillan, 2017; Des Marteaux et al., 2018).

1.1.2 Phenotypic plasticity of cold tolerance

Cold hardiness is phenotypically plastic in response to seasonal changes in environmental conditions (Jakobs et al., 2015; MacMillan et al., 2016), as both short and long-term cold exposure can enhance cold-tolerance (Chown and Sinclair, 2010). Rapid cold hardening, for example, improves cold tolerance almost immediately (i.e. minutes to hours), as insects respond rapidly to low temperature stress (Teets and Denlinger, 2013); this is advantageous in temperate regions where air temperatures can fluctuate by > 20 °C daily (Lee, 2010). Alternatively, cold tolerance can be enhanced after prolonged exposure (i.e. days to weeks) to low temperatures in the form of seasonal acclimatization (i.e. responses to natural changes in environmental conditions) (Colinet and Hoffman, 2012; Teets and Denlinger, 2013); this seasonal cold-hardening may be more important for sustaining cold tolerance over an entire overwintering period (Teets and Denlinger, 2013).

Many temperate insects enhance their cold tolerance (i.e. acclimatize) in response to seasonal changes in temperature and photoperiod (Lee, 1989; Teets and Denlinger, 2013). Similar improvements in cold tolerance are inducible in lab via acclimation (i.e. responses to lab controlled environmental conditions) under low temperatures or short photoperiods **(**Teets and Denlinger, 2013). For example, in *G. pennsylvanicus*, prior cold-acclimation lowers the critical thermal minimum, chill coma recovery time, and incidence of injury and mortality following cold shock (Coello Alvarado et al., 2015). Additionally, seasonal acclimation aids in maintaining homeostasis and cell integrity at low temperatures, protecting against chill-injury (Des Marteaux et al., 2017, 2018), which appears to be important for enhancing cytoskeletal stability and improving survival after cold exposure ; Kim et al., 2006; Des Marteaux et al., 2017). By contrast, overwintering larvae of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), accumulate high levels of trehalose after acclimation to low temperatures, resulting in reduced SCPs and prolonged survival at 5 °C relative to non-acclimated larvae (Khani et al., 2007). In this example, trehalose may act as a physical and/or chemical protectant that maintains cellular integrity through the protection of cell membranes and proteins (Behm, 1997; Khani et al., 2007). Moreover, the accumulation of heat shock proteins in diapausing (see section 1.4) pupae of the onion maggot, *Delia antiqua* (Diptera: Anthomyiidae), is correlated with increased cold hardiness by promoting

membrane stability and repressing actin depolymerization (Kayukawa and Ishikawa, 2009; King and MacRae, 2015).

1.2 Insect water balance

Given their small size and high surface area-to-volume ratio, insects may experience substantial water loss. Changes in an insect's overall water content reflect differences between the rate at which they gain water, and the rate at which they lose it (Harrison et al., 2012). Insects have developed three primary strategies to resist desiccation: they can a) restrict water loss, b) accumulate more total water in preparation for overwintering, and c) tolerate greater overall water loss (Gibbs et al., 1997; Bazinet et al., 2010).

Insects are exposed to cold, dry air throughout winter, which exacerbates desiccation stress. In cold microhabitats the vapour pressure of ice is less than that of water at the same temperature; as temperature decreases, water will tend to transfer from unfrozen insects to surrounding ice, promoting desiccation (Danks, 2000). Water loss is reduced by controlling both cuticular and respiratory water loss. For example, the periodic closing of spiracles associated with some gas exchange patterns reduces respiratory water loss; however, this loss accounts for a small portion of total water loss in temperate environments (i.e. < 20%; Chown, 2002; White et al., 2007), and altering it has a minimal impact on overall water balance. Alternatively, cuticular water loss represents the major source of water loss for insects (>80%; , Hadley, 1994**)** which can be mitigated through the thickening of the cuticle, often through the accumulation of cuticular hydrocarbons (Nelson and Lee, 2004) or by modifying the cuticular hydrocarbon profile (Stinziano et al., 2015)

Insects can gain water by drinking, ingesting food, aerobic metabolism (i.e. metabolic water), and through specialized organs which allow for water to be extracted from water vapour (Danks, 2000; Harrison et al., 2012). Many herbivorous insects obtain most (if not all) of their water from the plants which they consume (Harrison et al., 2012). However, overwintering insects usually do not feed, resulting in reduced water intake. In *D. melanogaster,* flies which are selected for desiccation-resistance have lower water loss rates, and contain more water and glycogen relative to non-desiccation-selected flies (Gibbs et al., 1997). Glycogen, which hydrogen-binds 3 - 5 times

it's mass in water, can be metabolised to provide increased water stores under desiccating conditions, resulting in increased desiccation resistance (Gibbs, 2002). Hydrogen-bound water may be an important source of water throughout the overwintering period, as many overwintering insects accumulate glycogen reserves prior to diapause (Hahn and Denlinger, 2011), which can be metabolised if insects become water-stressed.

Insects can tolerate varying amounts of body water loss; most species can tolerate losing 30 - 40 % (Benoit, 2010), while species such as the Antarctic midge, *Belgica antarctica* (Diptera: Chironomidae), can tolerate losing > 70 % (Benoit et al., 2007). Species which can maintain water balance over prolonged periods of desiccation are, therefore, considered desiccation tolerant. Increased body size has a considerable effect on desiccation resistance, as larger insects are generally capable of losing larger quantities of water (Hadley, 1994). For example, in the tropical beetle, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae), larger female beetles have greater initial water content, and can tolerate losing 4% more water than smaller males (Renault and Coray, 2004).

1.2.1 Phenotypic plasticity of water balance

Like cold tolerance, water balance in insects is seasonally plastic. Overwintering insects may modify their water balance by reducing their water content and/or water loss rates. Dormant insects often have reduced water content resulting from fat accumulation and metabolic adjustments (Danks, 2000); by reducing water content, insects reduce the likelihood of extracellular ice formation (Ring and Danks, 1994). To reduce overall water loss rate, insects may suppress their metabolic rate, resulting in lower respiratory water loss. For example, pupae of the corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae), overwinter in diapause, in which metabolic rate suppression reduces respiratory water loss and, consequently, their water loss rate (Benoit et al., 2015). However, limiting cuticular water loss is likely the most efficient means of maintaining water balance while overwintering, and can be achieved by reducing cuticular permeability. Possible mechanisms include increasing the saturation and/or reducing the number of methyl chains in cuticular lipids, as in *D. melanogaster* (Stinziano et al., 2015), or increasing the quantity of cuticular hydrocarbons like larvae of the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae) (Nelson and Lee, 2004).

1.3 Overwintering energetics

Overwintering insects generally do not feed and must rely on energy stores to fuel both winter survival, and development and reproduction in spring (Lester and Irwin, 2012; Sinclair, 2015); as a result, overwintering insects are at risk of starvation and/or reduced fitness (Sinclair and Marshall, 2018). Temperature influences metabolic rates, and therefore influences energetic depletion which is problematic for non-feeding insects (Williams et al., 2015a). As global temperatures increase and winters continue to get warmer, it is hypothesized that overwintering insects face a greater risk of mortality resulting from increased energy consumption (Williams et al., 2015b).

In preparation for diapause and/or overwintering, many insects will accumulate lipid stores - predominantly as triacylgylcerols (Marshall et al., 2014; Sinclair and Marshall, 2018) - and carbohydrate stores as glycogen in the fat body (Han and Bauce, 1998; Hahn and Denlinger, 2011). Triacylglycerols provide the highest caloric content of available energy stores (per gram), and glycogen is converted to glucose and/or trehalose to fuel catabolic processes (Storey and Storey, 1991; Hahn and Denlinger, 2011). Carbohydrates can be further metabolised to produce polyol cryoprotectant molecules such as glycerol and sorbitol (Storey and Storey, 1991). Insects may also accumulate amino acids in the form of specialized storage proteins, which may be used to fuel post-overwintering development (depending on overwintering life stage; Hahn and Denlinger, 2011). Starved insects appear to switch from carbohydrate- to lipid-fuelled metabolism during early stages of starvation, indicating the importance of accumulating large stores of both lipids and carbohydrates (Sinclair and Marshall, 2018). In general, overwintering fuel selection is dependent in part on life-history trade-offs and physiological necessities; species that cannot replenish lipid reserves post-overwintering may selectively consume carbohydrate stores, while insects which use carbohydrate stores for production of cryoprotectants may selectively consume lipid stores (Sinclair and Marshall, 2018).

Insects employ both physiological and behavioural adaptations to mitigate exposure to environmental stress and to mitigate energy depletion. Many insects overwinter in diapause (section 1.4) which promotes suppressed metabolic rate and reduces overall energy expenditure (Tauber et al., 1986). The nonlinear relationship between temperature and metabolic rate means

that energy use is greater under fluctuating thermal regimes (Williams et al., 2015a). Because metabolic rate increases exponentially with temperature, warm conditions and fluctuating temperatures will disproportionately increase energy consumption in insects (Williams et al., 2012b; Colinet et al., 2015; Sinclair, 2015). As a result, many insects overwinter in cool, protected microhabitats that buffer thermal variation, thus reducing the risk of energy depletion. For example, freeze-tolerant *P. isabella* overwinter in exposed habitats where exposure to low temperatures (and subsequent freezing and thawing) leads to energy savings (Marshall and Sinclair, 2012), while insects which burrow such as the Eastern subterranean termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae), experience warmer temperatures, but are protected from extreme high temperatures in the fall and autumn, and extreme low temperatures in the winter (Clarke et al., 2013).

1.4 Diapause

Diapause is a state of programmed dormancy characterized by metabolic suppression, arrested development, and reduced activity (Koštál, 2006). Diapause initiation may be obligate and occur regardless of environmental cues; in the orchard mason bee, *Osmia lignaria* (Hymenoptera: Megachilidiae), individuals always enter diapause after adult eclosion in the fall, and terminate diapause once optimal temperature conditions are met (Sgolastra et al., 2010). Alternatively, many insects enter facultative diapause which is regulated by environmental cues (e.g. photoperiod and temperature; Koštál, 2006; Lester and Irwin, 2012). Adults of the southern green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae), enter diapause in response to shortened days (i.e. reduced photoperiod; Musolin and Numata, 2003) whereas the oblique-banded leafroller, *Choristoneura rosaceana* (Lepidoptera: Tortricidae), enters diapause in response to reduced photoperiod and exposure to fluctuating temperatures, particularly if the higher range of temperatures experienced in a 12 h period exceeds 25 °C (Gangavalli and Aliniazee, 1985).

Stress tolerance is often enhanced in diapause (Irwin and Lee, 2012; Coleman et al., 2014; Benoit et al., 2015). Prior to diapause induction, insects may accumulate lipids and carbohydrate reserves to fuel overwintering energetic demands and promote stress tolerance (Tauber et al., 1986), and insects stop development after diapause initiation, reducing the rate of energy depletion (Koštál, 2006). Moreover, diapause is associated with suppressed metabolism (Koštál, 2006)

which limits overall energy expenditure; this depression, coupled with low winter temperatures, promotes the economic use of energy reserves (Hahn and Denlinger, 2007), and reduces water loss. The degree of metabolic suppression varies in insects; for example, adult monarch butterflies, *Danaus plexippus* (Lepidoptera: Nymphalidae), sustain flight capacity and show little metabolic reduction while overwintering in reproductive diapause (Herman, 1981; Chaplin and Wells, 1982), whereas diapausing pupae of the flesh fly, *Sarcophaga argyrostoma* (Diptera: Sarcophagidae), reduce their metabolic rate by approximately 90 % compared to non-diapausing pupae (Denlinger, 1972). Additionally, cold hardiness is often an integral component of the diapause program, and diapause often enhances an insect's capacity to cold harden (Denlinger, 1991). Diapause and diapause-related metabolic suppression can be correlated with accumulating cryoprotectants (e.g. glycerol, sorbitol) and SCP depression (Milonas and Savopoulou-Soultani, 1999), likely enhancing cold tolerance. The diapause program therefore plays a multi-faceted role in promoting insect stress tolerance and enhancing overwintering survival.

1.5 The brown marmorated stink bug, *Halyomorpha halys*

Halyomorpha halys (Hemiptera: Pentatomidae), the brown marmorated stink bug, is an invasive agricultural pest native to subtropical and temperate areas of southeastern Asia (i.e. China, Japan, Korea, and Taiwan; Hoebeke and Carter, 2003). Initial establishment in North America likely occurred in 1996 in Allentown, Pennsylvania (Hoebeke and Carter, 2003). Since then, *H. halys* has rapidly expanded its range, and now occurs in >40 states, several European countries (including Austria, France, Georgia, Germany, Italy, Russia, Spain and Switzerland; Kriticos et al., 2017), parts of South America (Faúndez and Rider, 2017), and in three Canadian provinces (British Columbia, Ontario, and Quebec) (Fogain and Graff, 2011; Gariepy et al., 2014a, 2014b; Abram et al., 2017). Moreover, bioclimatic models predict that *H. halys* will be able to establish in New Zealand and Australia (Haye et al., 2015; Zhu et al., 2012; Zhu et al., 2017). The movement of *H. halys* into new regions is likely attributable to its hitchhiking abilities; *H. halys* have been transported from Asia to several locations globally by (but not limited to) aircraft cargo, packing crates, vehicles, and personal luggage (Hoebeke and Carter, 2003; Gariepy et al., 2015). The ability of *H. halys* to successfully establish within a new region is a function of the interactions between climatic tolerance, host availability, and anthropogenic influences (Bakken et al., 2015; Venugopal et al., 2016).

Halyomorpha halys is polyphagous, attacking >200 plant hosts including economically important crops such as apples, peaches, peppers, sweet corn, and soybeans (Nielsen and Hamilton, 2009; Philips et al., 2016). *Halyomorpha halys* damages crops throughout the entirety of the growing season; adults emerging from overwintering sites feed on early- and mid-season apple crops (Funayama, 2004), with the most damage occurring during mid-season crop development (Nielson and Hamilton, 2009). *Halyomorpha halys* have a stylet mouthpiece, which they use to pierce plant tissue; digestive enzymes within the saliva cause tissue necrosis, rendering the crop unmarketable (Rice et al., 2014; Costi et al., 2017). *Halyomorpha halys* feeding damage resulted in $37 million in losses to mid-Atlantic apples in the USA in 2010 (Leskey et al., 2012; Rice et al., 2014), and inflicted crop damage in 50% of commercial pear orchards in Italy in 2015, just three years after it was first detected in the country (Maistrello et al., 2017). *Halyomorpha halys* poses a significant threat to Canadian agricultural productivity given its establishment in close proximity to important fruit tree production areas (i.e. Niagara Peninsula and Okanagan Valley) (Gariepy et al., 2014a; Abram et al., 2017).

Halyomorpha halys overwinter as adults in a reproductive diapause, induced by shortened photoperiods (Niva and Takeda, 2003). In fall, adult *H. halys* aggregate and overwinter in protected microhabitats including natural habitats (e.g. standing dead trees) and homes and residential structures, giving rise to their nuisance pest status (Inkley, 2012; Lee et al., 2014). Cira et al. (2016) found that the SCP of *H. halys* is seasonally plastic and can be depressed to -17 °C in the winter, but adults will die after exposure to temperatures between -5 and -10 °C, indicating chill-susceptibility. However, Cira et al. (2016) failed to measure *H. halys* LLT; given that they are chill-susceptible, it is inappropriate to describe *H. halys* cold hardiness using SCP alone. By contrast, acute exposure of diapausing *H. halys* to temperatures as low as -4 °C has no immediate or long-term effects on post-diapause survival, and appear to increase fecundity (Lowenstein and Walton, 2018). Moreover, adults emerging from overwintering sites in Japan weigh significantly less than individuals preparing to overwinter (Funayama, 2012), which is likely the result of energy depletion and/or desiccation. While some information is available on *H. halys* cold tolerance, there remains a knowledge gap regarding the importance of other stressors - desiccation and energy depletion in particular – in determining *H. halys* overwintering success, and the importance of diapause in enhancing stress tolerance (if at all).

1.6 Objectives

In this thesis I addressed three main objectives in relation to *H. halys* overwintering:

1. I will determine the relative importance of low temperatures, desiccation and energy depletion in determining *H. halys* overwintering success by taking regular measurements of survival and stress tolerance in overwintering field colonies.
2. I will determine if *H. halys* stress tolerance changes seasonally by comparing tolerance of low temperatures, desiccation, and energy depletion in winter- and summer-acclimatized field colonies, and in diapause and non-diapause-acclimated lab colonies.
3. I will determine the role of diapause in enhancing *H. halys* tolerance to low temperatures, desiccation, and energy depletion using diapause and non-diapause-acclimated lab colonies.

2 Methods

2.1 Insect collection

I collected adult *H. halys* in September and October of 2016, and from May to September in 2017 at field sites in London, Ontario. Insects were collected using pheromone pipe traps (Figure 2.1) baited with commercial Trécé *H. halys* and green stink bug (*Acrosternum hilare;* Hemiptera: Pentatomidae) attractants (Trécé, Inc., Adair, OK, USA). I emptied traps twice weekly at each trap site in London. . Additionally, I performed visual surveys at trap sites; I surveyed host trees (e.g. *Rhamnus cathartica, Acer negundo, Acer saccharum*) in urban environments and host crops (e.g. corn, soy, and apples) in agricultural environments. Upon collection, I placed insects in a styrofoam cooler and transferred them directly to an insect rearing facility at Agriculture and Agri-Food Canada, London Research and Development Centre (AAFC-LoRDC). Insects collected in fall 2016 and 2017 were used to populate overwintering field colonies from October 2016 to April 2017, and October 2017 to April 2018, respectively (section 2.3).

Figure 2.1 Experimental PVC pipe trap baited with commercial Trécé *Halyomorpha halys* and *Acrosternum hilare* attractants. Traps were placed in agricultural (i.e. corn, soy, and apple) and urban (i.e. residential) field sites in London, Ontario. Total trap height is 1.2 m.

2.2 Rearing conditions

I reared *H. halys* in mesh insect cages (47.5 cm × 47.5 cm × 47.5 cm; MegaView Science Co Ltd, Talchung, Taiwan**)** in a temperature-controlled growth chamber at 24 ± 1 °C and 50 ± 5 % relative humidity under long day-length (16:8 L:D). Insects were reared with potted red kidney bean plants (variety "California" ; Stokes Seeds, Thorold, ON, Canada) and fed a diet of organic romaine lettuce, carrots, apples, and raw peanuts. I collected egg masses three times per week and randomly divided them to treatment groups (diapause and non-diapause). Adults from the rearing colony were used in subsequent overwintering field colonies and for comparison to insects reared under diapause conditions.

To determine the role of diapause in driving changes in stress tolerance, I reared a separate population of *H. halys* adults under diapause-inducing conditions at AAFC-LoRDC. I collected egg masses from the non-diapause colony weekly and reared until hatch. First-instar nymphs were transferred to a piece of lettuce in an insect rearing cage in a temperature controlled rearing cabinet at 24 ± 1 °C and 50 ± 5 % RH and reared under short day length conditions (8L:16D). Diapause insects were reared with the same diet as the non-diapause colony. The diapause rearing conditions were not meant to reflect the overwintering conditions experienced by *H. halys*, but simply to induce diapause. All experiments involving diapausing and non-diapausing adults were performed on adults 2-3 weeks after eclosion.

2.3 Experimental design

In mid-October 2016 and 2017, I assessed survival of adult stink bugs in protected and non-sheltered overwintering microhabitats, in addition to characterizing their stress tolerance. In winter 2016/17 I transferred approximately 500 adult stink bugs into one of nineteen 960 mL cylindrical plastic BugDorm containers (Megaview Science Co Ltd) along with three rolled brown paper bags (as sheltering substrate) and a fitted mesh lid. Containers were then placed into one of two large plastic containers; one large container with 100 stink bugs (ten adults per 960 mL BugDorm) was placed in a shaded suburban garden in London, ON (42°59'N, 81°17'W, 251 m elevation), while a second large container containing approximately 350 stink bugs (divided into nine 960 mL BugDorms) was placed in a non-heated garage in London, ON. Every two weeks, I transferred one

960 mL container from the garden to the lab and recorded survival (Figure 2.2A); this was repeated ten times over a 20 week sampling period (Figure 2.2B). Additionally, I assessed the stress tolerance (see sections 2.4 - 2.6) of garage-acclimatized stink bugs at three time points (Figure 2.2A,B) – early winter (late November), mid winter (early January) and late winter (early April; see sections 2.4, 2.5, and 2.6). Stink bugs were held in an incubator (MIR153, Sanyo, Bensenville, Il, USA) at 12 ± 1 °C for a maximum of 48 h before analysis. In winter 2017/18, I repeated this overwintering study with slight modifications; I placed 200 stink bugs into a large container within the suburban garden (i.e. 20 adults per 960 mL BugDorm), and I assessed stress tolerance at three different time points – early December, early February, and early April - using approximately 450 stink bugs sampled from the garage. I placed the probes of a HOBO Pro v2 U23-03 data logger (Onset Computer Corporation, Bourne, MA, USA) in each large container (i.e. garden and garage), and recorded ambient temperature every 30 minutes from 31 October 2016 to 4 March 2017, and from 18 October 2017 to 3 April 2018.

To assess the effect of diapause on *H. halys* stress tolerance, I compared responses to stress tolerance in laboratory-reared diapause and non-diapause males and females. In doing so, I measured tolerance to low temperatures (i.e. SCP, LT_{50}, cold tolerance strategy, and hemolymph osmolality; section 2.4), desiccation (i.e. water content, desiccation time, water loss rate and water content at death; section 2.5), and energy depletion (i.e. lipid and carbohydrate stores; section 2.6). In addition, I used flow-through respirometry to measure metabolic rate, cuticular water loss, and respiratory water loss (section 2.7) in both treatment groups, and dissected males and females of each treatment group to assess reproductive development (section 2.8).

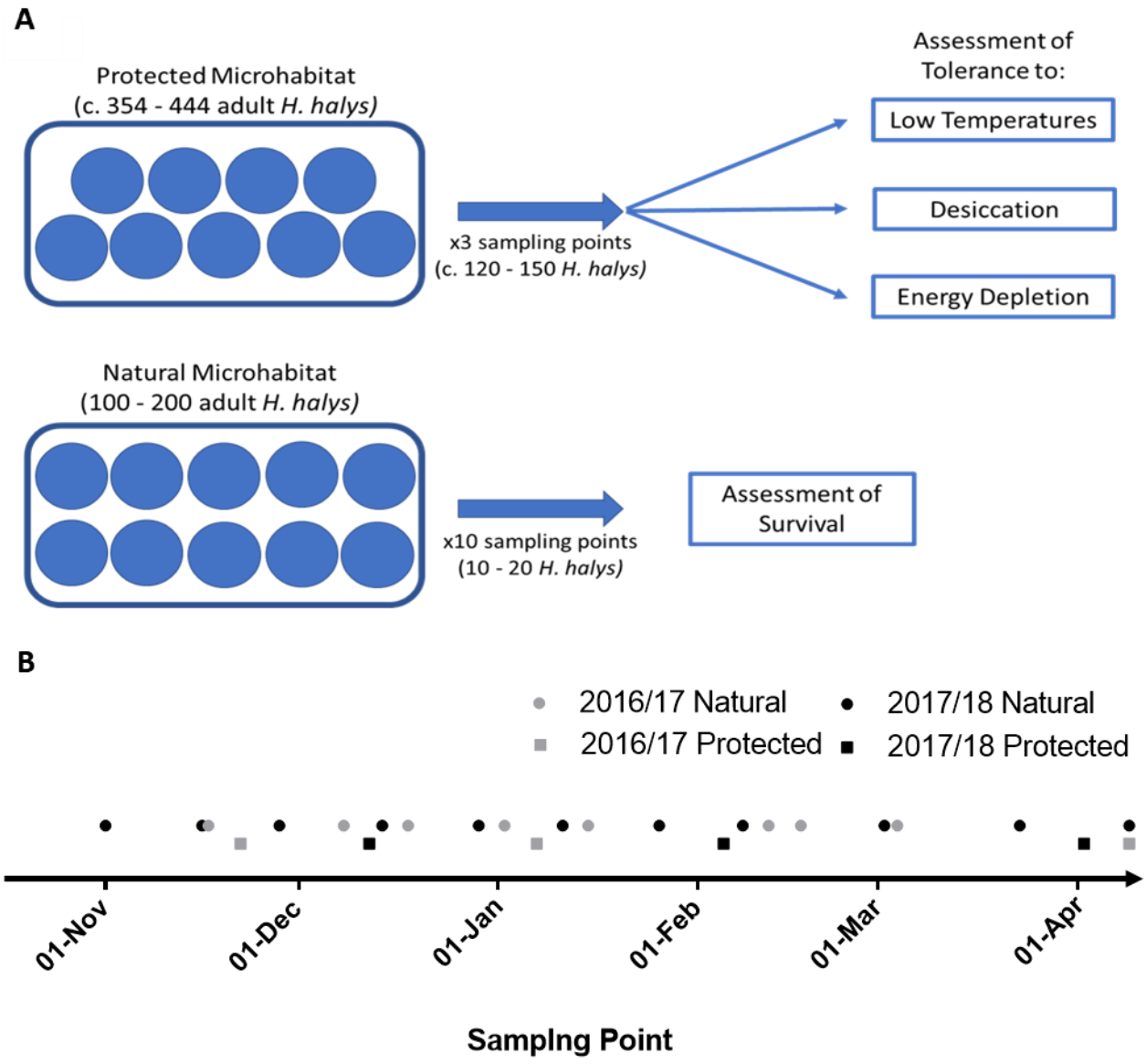

Figure 2.2 Overwintering field study experimental design (A) and sampling points from winter 2016/17 and 2017/18 (B). Adult *H. halys* from a protected (garage) microhabitat in London, ON were transferred to the University of Western Ontario (UWO; London, ON) at three time points during both winter 2016/17 and 2017/18 to measure *H. halys* stress tolerance. Additionally, adult *H. halys* were transferred from a natural (suburban harden) microhabitat (London, ON) to UWO at ten sampling points during winter 2016/17 and 2017/18 to assess survival.

2.4 Cold tolerance

Individual adult *H. halys* from each sampling period were transferred to 1.7 mL microcentrifuge tubes and held in contact with a 36-AWG type-T thermocouple (Omega, Laval, Quebec, Canada). The tubes were transferred to an aluminum cooling block and cooled with methanol (100 %) circulated by a refrigerated bath (Lauda Proline 855, Wurzburg, Germany); insects were then cooled to -30 °C at -0.5 °C/min. Thermocouples were connected to PicotechTC-08 interfaces and data acquired by PicoLog software (Pico Technology, Cambridge, UK, Version 5.25.3). The SCP was the lowest temperature prior to the exotherm. I compared the SCPs of different treatments and sampling points using ANCOVA in R version 3.3.3 (R Development Core Team, 2017) with fresh mass as a covariate.

I determined cold tolerance strategy by assessing *H. halys* survival after cold exposure and ice formation (Sinclair et al., 2015). To determine cold tolerance strategy, individuals of each sex (male and female) were placed into individual 1,7 mL microcentrifuge tubes in contact with thermocouples and cooled as described for SCPs. Once half the stink bugs had frozen (indicated by exotherms), all individuals were removed from the cooling block and transferred individually to 6-well plates with a moist cotton ball. Survival was assessed after 24 h at room temperature (c. 23 ±1 °C). Individuals that died from chilling injuries unrelated to freezing were considered chill-susceptible, while those that died upon freezing (i.e. mortality only seen at the SCP) were considered freeze-avoidant, and those which survived freezing were considered freeze-tolerant (Table 1.1).

I determined the LLT of *H. halys* from each sampling point through acute exposure (1 h) to low temperatures. Groups of 4-5 adult *H. halys* were placed individually into 1.7 mL microcentrifuge tubes, transferred to an aluminum cooling block, and held at a temperature ranging from -5 °C to -20 °C (resulting in 0 to 100 % mortality) for 1 h using a methanol circulator. Temperatures were recorded using thermocouples connected to PicotechTC-08 interfaces (Pico Technology). After 1 h, individuals were removed from the cooling block and transferred to a 6-well microplate containing a moist cotton ball; I assessed survival after 24 h at room temperature. The LT_{50} (lower temperature at which 50 % of insects die after 1 h of exposure) was calculated using a generalized linear model with a binomial error distributions and logit link function in R

(version 3.3.3). I calculated 95 % confidence intervals at each sampling point, which I used to compare LT_{50} values based on whether confidence intervals overlapped; if they overlapped, the lethal temperatures were not considered to differ significantly. I calculated 95 % confidence intervals using the *qnorm* function in R (version 3.3.3). I could not calculate 95 % percent confidence intervals fortwo sampling points, however, as all adults died between -5 °C and -10 °C during summer 2017, and between -17.5°C and -20 °C at early winter 2017/18, and I could therefore not use the *qnorm* function; these points were therefore left out of the analysis.

I measured hemolymph osmolality and thermal hysteresis using a nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand) following methods outlined by Crosthwaite et al. (2011). I collected hemolymph from 5-10 adults at each seasonal sampling point via centrifugation (1986 x g, 10 min) after removing the legs from each individual; hemolymph samples were emptied into a 1.7 mL microcentrifuge tube, overlaid with immersion oil, snap frozen in liquid nitrogen and stored at -80 °C until analysis. I measured hemolymph melting point by loading small droplets of hemolymph into small wells filled with immersion oil under a microscope and cooling the droplets until frozen. Droplets were then warmed slowly until a single crystal remained suspended in the well; the temperature at which this crystal melted was recorded as the melting point. Melting point was used to determine osmolality, with one mole of solute resulting in a 1.86 °C decrease in melting point (Crosthwaite et al., 2011). To detect thermal hysteresis, I refroze samples in the same fashion as melting point determination, and warmed samples to a temperature just below their melting point with a single ice crystal remained. The crystals were stabilized for one minute, then very slowly cooled until growth was observed. The temperature at which crystal growth began was taken as the hysteresis freezing point; thermal hysteresis was then calculated as the difference between melting point and hysteresis freezing point (Crosthwaite et al., 2011).

2.5 Desiccation

I calculated water content and water loss rate of adult *H. halys* using gravimetric methods as outlined by Bazinet et al. (2010). Individual *H. halys* were weighed (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA; d=0.1µg) to determine fresh mass (FM), then placed into empty 35 mL plastic fly vials and restricted to the lower portion using a foam stopper. Vials were filled with 4-10 mesh silica gel desiccant (Avantor Performance Materials LLC, Centre Valley, PA, USA)

and sealed with parafilm (Figure 2.3). Once individuals had died (i.e. did not respond when prodded), they were reweighed to determine mass at death and dried at 60 °C for a minimum of two days, after which they were weighed again to determine dry mass. Water content and water loss rate were determined gravimetrically using the following equations:

$$WC = FM - DM \quad (1)$$

$$WLR = \frac{(WC - WCD)}{Time} \quad (2)$$

where WC is water content, DM is dry mass, WLR is water loss rate, and WCD is water content at death. I compared water content and water loss rate for each sampling point using ANCOVA, with dry mass as a covariate (R version 3.3.3).

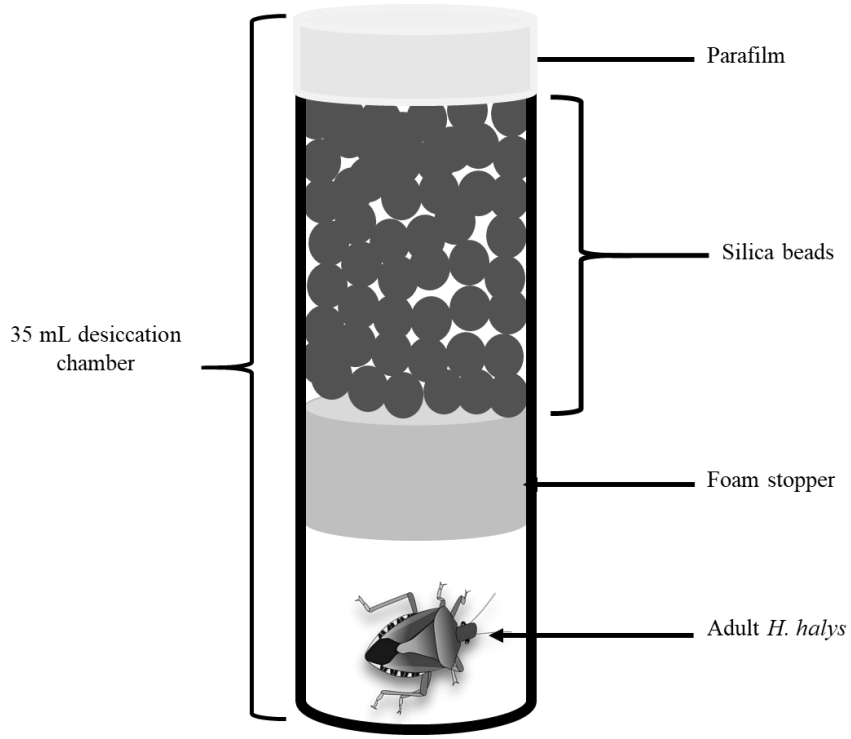

Figure 2.3 Schematic of a desiccation vial used to measure water content and water loss rate of *H. halys* gravimetrically. Adults were weighed prior to desiccation to determine fresh mass, after death to determine mass at death, and after drying in an oven (for two days minimum) to determine dry mass.

2.6 Energy depletion

To determine seasonal changes in energy stores, I quantified soluble protein, neutral lipids, and carbohydrates of individual *H. halys* from each sampling point. I weighed each insect to determine fresh mass, snap-froze them in liquid nitrogen, and dried individuals at 60 °C until no further mass loss was evident. Dried insects were reweighed to determine dry mass, and water content as described in equation 1. I homogenized dried insects with a Bullet Blender tissue homogenizer (Next Advance Inc, Troy, NY, USA) in 1.7 mL microcentrifuge tubes using zirconium silicate beads (0.7mm; Next Advance Inc). Homogenized tissue was added to 0.05 % Tween 20 in ddH_20 (10 µL 0.05 % Tween per 1 mg dry mass), frozen in liquid nitrogen, and stored at -80 °C until further analysis.

I quantified soluble protein concentration using the bicinchoninic acid (BCA) assay following Williams et al. (2011) and Gefen et al. (2006). 10 µL of each homogenised sample was transferred into 1 mL 0.05 % Tween 20, vortexed, loaded in 10 µL triplicates on microplates, and 200 µL of BCA reagent (50 parts BCA: 1 part 4% $CuSO_4$) was added to each well. Plates were incubated for 30 minutes at 37 °C, and absorbance was measured at 562 nm. Protein standards were included on each plate (0.025-0.75 $mg \cdot mL^{-1}$ bovine albumin serum, Thermo Scientific, Rockford, IL, USA). Soluble protein content (mg) was used as a proxy for metabolizing tissue (Williams, 2011; Williams et al., 2012a), and as a covariate in comparisons of lipid and carbohydrate content across time points (ANCOVA; R version 3.3.3).

I quantified lipid concentration gravimetrically using a modification of the Folch method for neutral lipid extraction (Folch et al., 1956). I transferred 20 µL aliquots of each homogenized sample (i.e. 2 mg of dry tissue) to 2.0 mL gas chromatography vials (Agilent Technologies, Santa Clara, CA, USA), which were then weighed. Samples were washed with 1.0 mL of chloroform on a plate shaker for 30 minutes. Chloroform was evaporated and samples were dried at 60 °C for 24 h. I repeated chloroform washes every 24 h for a total of three days, after which no further mass loss occurred. Lipid content was calculated as lipid free dry mass (LFDM) subtracted from initial tissue mass (i.e. mass of tissue in 20 µL aliquot). Total lipid content (mg) was compared across sampling points using ANCOVA, with soluble protein content as a covariate (R. version 3.3.3)

I quantified carbohydrates using a modification of the anthrone method (Carroll et al., 1956; Williams et al., 2012a). Prior to the assay, I diluted each homogenized sample by adding 10 µL of each to 100 µL 0.05 % Tween 20. 100 µL of each diluted sample was added to 100 µL 30 % KOH in test tubes, briefly incubated (100 °C, 20 min) to extract glycogen, and centrifuged (2000 × g, 10 min). I aliquoted 150 µL of the supernatant into 300 µL 95% ethanol, and precipitated glycogen by adding 7.5 µL Na_2SO_4. Samples were centrifuged (2000 × g, 10 min) and the resulting pellet dried at 100 °C; samples were reconstituted in 250 µL distilled water. I added 750 µL of cold anthrone reagent (0.05 % anthrone [w/v], 1 % thirourea [w/v], 28 % water [v/v], 72 % H_2SO_4 [v/v]) to each sample and standard (0.01 - 1.0 $mg \cdot mL^{-1}$ glycogen; Sigma Aldrich, Oakville, ON, Canada) and cooled in an ice bath for 10 min. Samples and glycogen standards were then incubated (100 °C, 15 min) and returned to the ice bath. I loaded samples and standards in triplicate on an acid-proof polypropylene 96-well plate, and measured absorbance at 620 nm. Total carbohydrate content (mg) was compared across sampling points using ANCOVA, with soluble protein content as a covariate (R version 3.3.3).

I expressed lipid and carbohydrate concentrations in $\mu g \cdot mg\ protein^{-1}$ and estimated whole-animal values by multiplying by dry mass. I calculated total energy content in Joules per adult assuming 39.3 $J \cdot mg\ lipid^{-1}$ and 17.6 $J \cdot mg\ carbohydrate^{-1}$ (Djawdan et al., 1998). Total energy content (J) was compared across time points using ANCOVA, with soluble protein as a covariate (R version 3.3.3).

2.7 Flow-through respirometry

To assess the thermal sensitivity of metabolic rate and metabolic suppression in diapausing and non-diapausing *H. halys*, I measured CO_2 production by individual adults from each treatment group (i.e. diapause and non-diapause) at 5, 10, 15, 20 and 25 °C following methods outlined by Williams et al. (2015a). Adults were weighed before and after each measurement. Air was scrubbed of CO_2 and water vapour using a Drierite-Ascarite-Drierite column. Dry, CO_2-free air was passed through glass chambers at 80 $mL \cdot min^{-1}$ by mass-flow valves (Sierra Instruments, Monterey, CA, USA) and a mass-flow controller (Sable Systems International [SSI], Las Vegas, NV, USA). I used an RM-8 multiplexer (SSI) to direct air flow through an empty reference chamber (baseline) or one of three chambers containing a single adult *H. halys*, then to an Li7000

infrared gas analyzer (LiCor; Lincoln, NE, USA) to measure CO_2 emission and water vapour. I controlled temperatures (± 0.5 °C) using a PELT-5 temperature-controlled cabinet (SSI) which housed all chambers. I recorded data on Expedata software using U12 interface (SSI). I corrected water and CO_2 measurements to the baseline recordings of the empty chamber and converted to the rate of CO_2 and water release ($mL \cdot min^{-1}$) using the equations:

$$\dot{V}CO_2 = \left(\frac{CO_2}{1000000}\right) x\ FR \quad (3)$$

where FR is the flow-rate of CO_2 free air in the chamber ($mL \cdot min^{-1}$), CO_2 is the difference in CO_2 entering and leaving the chamber (ppm), and:

$$\dot{V}H_2O = \left(\frac{H_2O}{1000}\right) x\ FR \quad (4)$$

where H_2O is the difference in H_2O entering and leaving the chamber (ppt; Lighton, 2008). I calculated mean CO_2 emission rate of each adult over a 1 h period after a minimum of 1 h acclimation in chamber and a 5 min washout. I compared $\log\dot{V}CO_2$ ($\mu L \cdot h^{-1}$) of diapausing and non-diapausing *H. halys* at 5, 10, 15, 20, and 25 °C using ANCOVA, with fresh mass (mg) as a covariate (R version 3.3.3). Additionally, I calculated Q_{10} - the thermal sensitivity of metabolic rate - of both diapausing and non-diapausing *H. halys* at a temperature range of 10 - 20°C, under the assumption that this range was not stressful to *H. halys* reared under either treatment. I calculated Q_{10} following the equation:

$$Q_{10} = (Rate2 \div Rate1)^{10 \div (T2 - T1)} \quad (5)$$

where Rate2 and Rate 1 are metabolic rate at 20 and 10 °C, respectively, and T2 and T1 are temperature at 20 and 10 °C, respectively. Cuticular water loss rate was calculated as the intercept of the regression of $\dot{V}H_2O$ against $\dot{V}CO_2$, while respiratory water loss was calculated as the difference between $\dot{V}H_2O$ and cuticular water loss rate (Gibbs and Johnson, 2004). I compared cuticular water loss rate and respiratory water loss rate ($mg \cdot hr^{-1}$) of diapausing and non-diapausing *H. halys* at 5, 10, 15, 20, and 25 °C using ANCOVA, with fresh mass (mg) as a covariate (R version 3.3.3).

2.8 Diapause Determination

To determine whether diapause-inducing conditions led to reproductive arrest in *H. halys,* I dissected ten males and females (2 - 3 weeks after eclosion) from each lab colony (i.e. diapausing and non-diapausing) and assessed reproductive tract development . Dissections were performed using methods adapted from Nielsen et al. (2017) and Jakobs (2014); I removed legs prior to dissection, and pinned stink bugs dorsally on a dissecting plate. Individuals were dissected laterally along the outer edges of the abdomen, and all abdominal segments were removed to expose the internal organs. In males, each testis was carefully removed and transferred to Ringer's solution on a microscope slide. This procedure was repeated on females, with the gut and ovaries (two per individual) removed and transferred to Ringer's solution. I took pictures of each pair of organs using a camera (Nikon digital sight DS-Fil, Tokyo, Japan) installed on a stereomicroscope (Nikon SMZ 1500, Tokyo, Japan). I assessed male reproductive development by measuring the length and width of each male teste in ImageJ (Version 1.8.0_122, ImageJ Developers), and calculating volume of each testis under the assumption that each was cylindrical. I compared length, width, and volume for each treatment using ANCOVA with fresh mass as a covariate (R Version 3.3.3). I described female reproductive systems as mature or immature using scoring systems outlined by Nielsen et al. (2017) and Penca and Hodges (2017). I used spermatheca presence (if visible) to categorize females as mated and determined development stage based on the presence of oocytes (if any), and measured the width of the vitellarium in relation to the germarium of each ovariole (if visible; Figure 2.3) in ImageJ (ImageJ Developers) to determine if females were diapausing or not (Nielsen et al., 2017; Penca and Hodges, 2017). I categorized females as diapausing if the germarium was wider than the vitellarium, and as non-diapausing if the vitellarium was wider than the germarium, and/or if mature oocytes were found (Penca and Hodges, 2017).

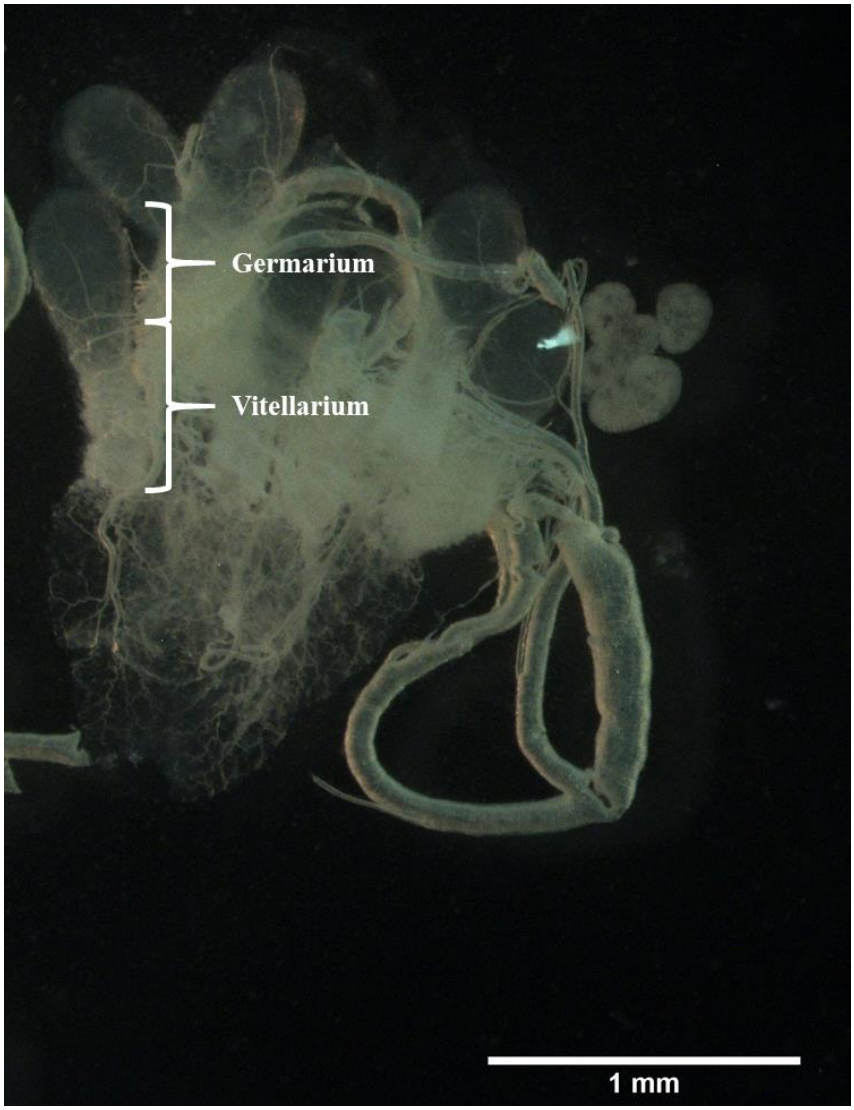

Figure 2.4 Germarium and vitellarium of a diapausing female *Halyomorpha halys* ovary. Ovaries from diapausing and non-diapausing female *H. halys* were removed and transferred to Ringer's solution on a microscope slide and photographed using a camera (Nikon digital sight DS-Fil) installed on a stereomicroscope (Nikon SMZ 1500).

3 Results

3.1 Overwintering field colonies

In winter 2016/17, the minimum temperature experienced by *H. halys* overwintering in natural and protected sites was -13.6 °C on 5 January, and -4.4 °C on 7 January, respectively (Figure 3.1A). In winter 2017/18, the minimum temperature experienced by *H. halys* overwintering in natural and protected sites was -18.9 °C on 7 January, and -5.0 °C on 14 December, respectively (Figure 3.1B). In both 2016/17 and 2017/18, *H. halys* were exposed to similar ambient temperatures while overwintering indoors, however, adults overwintering outdoors in 2017/18 experienced a colder winter than 2016/17 (Figure 3.1B).

In 2016/17, *H. halys* survival in protected sites declined over time (Figure 3.2), with half of sampled individuals alive in early-January, and less than a quarter alive by the last sampling point in early-March. By contrast, only one individual was found alive outdoors in March. In 2017/18, I observed a similar pattern in overwintering colonies; approximately half of the individuals sampled from the protected overwintering site were alive in early-February, while more than one-third were alive at the final sampling point in early-April. However, no live individuals were recovered outdoors after 29 December 2017, indicating that mortality was greater in insects overwintered outdoors (Figure 3.2).

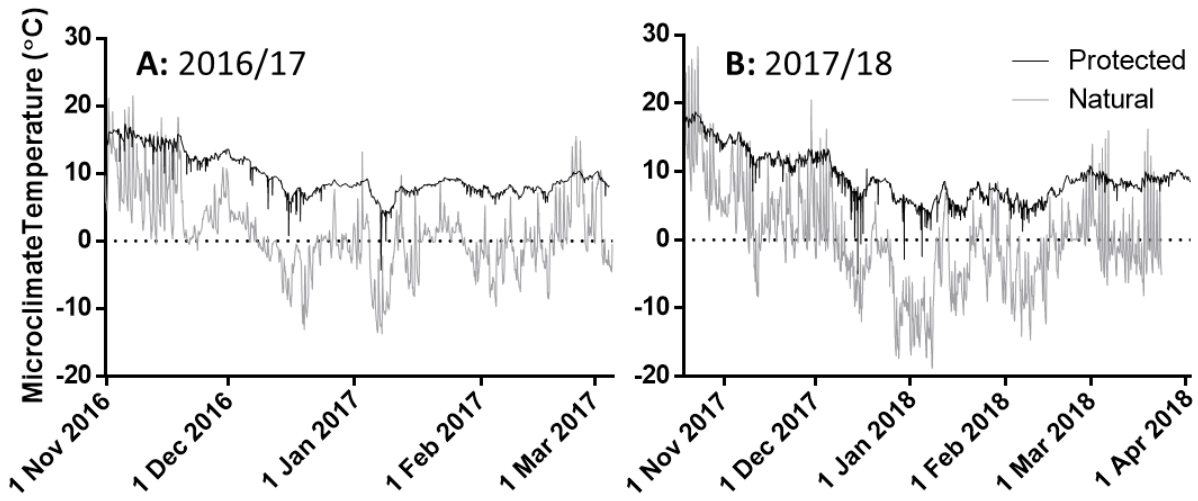

Figure 3.1 Microclimate data from natural (outdoor) and protected (indoor) sites in London Ontario collected in winter 2016/17 (A) and winter 2017/18 (B). Data are ambient air temperatures recorded using HOBO Pro v2 U23-03 data logger probes placed in each large container (i.e. protected and natural).

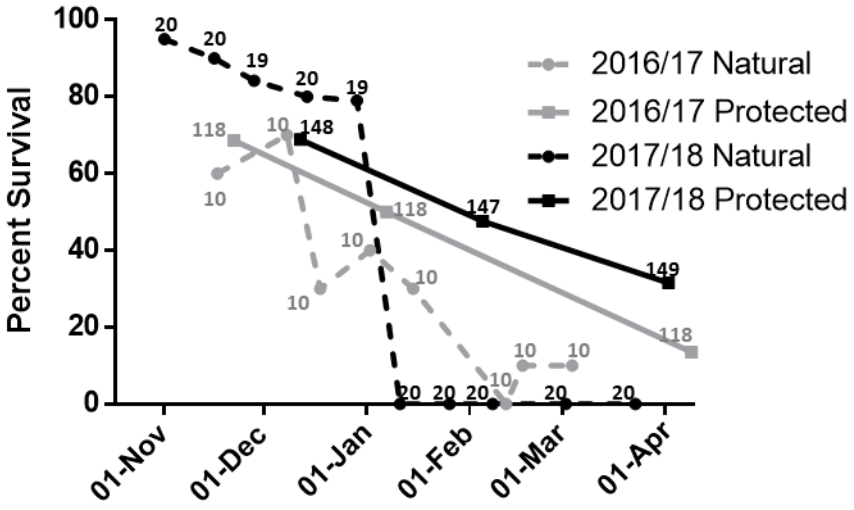

Figure 3.1 Survival of *Halyomorpha halys* overwintering in natural (outdoor) and protected (indoor) sites in winter 2016/17 and 2017/18. Adults overwintering in natural sites were sampled at ten samplings points during winter 2016/17 and 2017/18, and adults overwintering in protected sites were sampled at three sampling points. Numbers above each point indicate the total number of individuals (live and dead) collected at each sampling point. Dashed lines represent individuals sampled from the natural overwintering sites in winter 2016/17 (grey) and 2017/18 (black). .

3.1.2 Cold tolerance

The SCPs of individual adult *H. halys* ranged from -5.3 °C in summer 2017 to -17.5 °C in winter 2017/18 and were lower on average in the winter compared to summer ($F_{9,62} = 38.44$, $p < 0.001$; Figure 3.3). I found limited overlap in SCP between summer and winter-collected *H. halys*, as 100% of summer collected *H. halys* froze at 12.5 °C or higher (Figure 3.4). Moreover, males had lower SCPs compared to females ($F_{1,624} = 34.59$, $p < 0.001$; Figure 3.3); there was, however, no interaction between sex and season. *Halyomorpha halys* from all seasonal time points were unable to tolerate internal ice formation, suggesting that they are not freeze-tolerant. Additionally, when groups of individuals were cooled and half of them were frozen, more than half of the individuals that did not freeze also did not survive, suggesting that *H. halys* are chill-susceptible.

Cold-hardiness of *H. halys* was seasonally plastic. Adults depressed both their SCP and their LT_{50} in the winter; SCP was depressed from an average of -7.4 °C in the summer to -15.4 °C in the winter (Figures 3.3, 3.4), while LT_{50} was depressed from -5.3 °C during summer to as low as -17.5 °C in the winter (Figure 3.5). While I was unable to calculate 95 % confidence intervals for summer-sampled *H. halys* (Table 3.1), it is evident that LT_{50} at this time point is much lower compared to overwintering adults. Additionally, hemolymph osmolality was generally greater in winter compared to summer ($F_{6,42} = 5.06$, $p < 0.001$), however there was no difference in osmolality between summer 2018 and winter 2016/17 sampling points, or late winter 2017/18 ($p > 0.24$; Figure 3.6). Moreover, I found no differences in thermal hysteresis across sampling points ($F_{6,42} = 2.21$, $p = 0.064$).

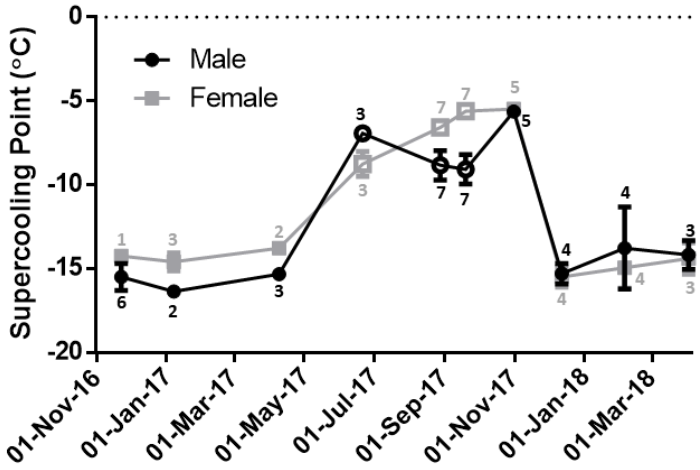

Figure 3.3 Seasonal variation in *Halyomorpha halys* supercooling point. Supercooling point was determined by cooling adults to -30 °C (in contact with a 36-AWG type-T thermocouple) and recording the lowest temperature prior to the exotherm. Results shown are mean SCP (± SEM) of n = 1 – 7 adult male and female *H. halys* compared across sampling points using ANCOVA with fresh mass as a covariate (numbers adjacent to each data point indicate sample size). Filled and open symbols indicate winter and summer sampling points, respectively.

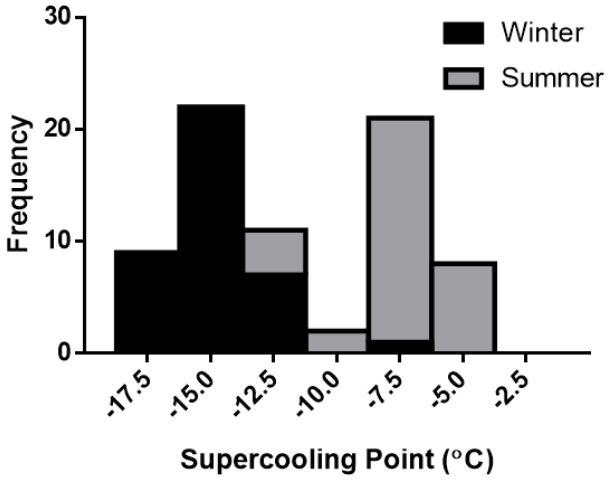

Figure 3.4 Observed seasonal variation in supercooling points of adult *Halyomorpha halys* collected during winter 2016/17 and 2017/18, and summer 2017.

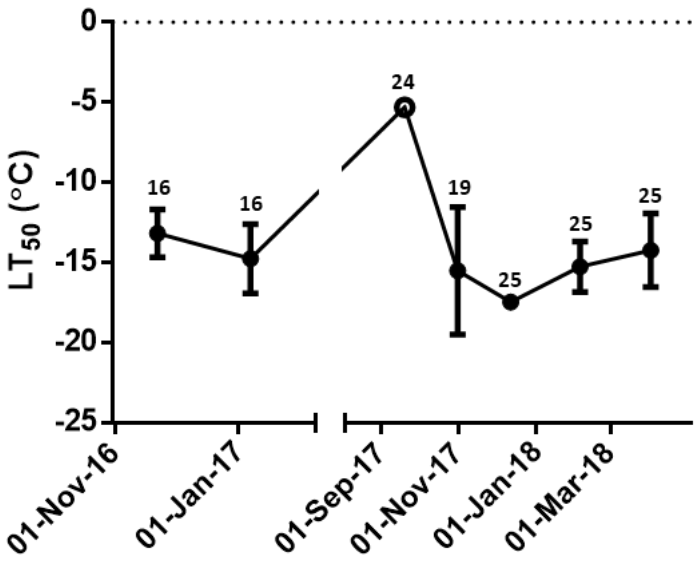

Figure 3.5 Seasonal variation in LT_{50} of *Halyomorpha halys* sampled in 2017 and 2018. Groups of 4 - 5 adult *H. halys* were exposed to a single temperature ranging from -5 °C to -20 °C for 1 h. The temperature at which 50 % of adults were dead (LT_{50}) was calculated with a generalized linear model (with a binomial error distributions and logit function). All points are shown as LT_{50} ± 95 % CI (except for late summer 2017 and early winter 2017/18 because 95 % CI could not be calculated) of n = 16 - 25 adult *H. halys* pooled at each sampling point (numbers adjacent to each data point indicate the number of individuals pooled at each sampling point). Filled and open symbols indicate winter and summer sampling points, respectively. 95 % confidence intervals were calculated using the *qnorm* function in R.

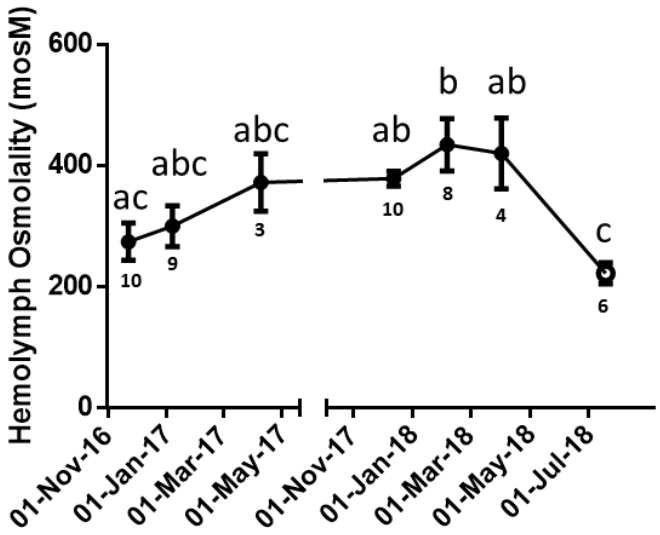

Figure 3.6 Seasonal variation in hemolymph osmolality of adult male and female *Halyomorpha halys*. Osmolality was determined by cooling droplets of hemolymph in a nanolitre osmometer (Otago Osmometers) until frozen, before being warmed until a single crystal remained. The melting point of the crystal was used to determine osmolality, as one mole of solute results in a 1.86 °C increase in melting point (Crosthwaite et al., 2011). All points are shown as mean hemolymph osmolality (± SEM) of n = 4 – 10 adults per sampling point (numbers adjacent to each data point indicate sample size). Points which do not share the same letter are statistically different ($p < 0.05$). Filled and open symbols indicate winter and summer sampling points, respectively.

Table 3.1 Lethal temperatures (LT_{50}) of overwintering and field-collected *Halyomorpha halys*. Groups of 4 - 5 adult (N = 20 - 25 total) *H. halys* were exposed to a single temperature ranging from -5 °C to -20 °C for 1 h. The temperature at which 50 % of adults were dead (LT_{50}) was calculated with a generalized linear model (with a binomial error distributions and logit function). 95 % confidence intervals were calculated using th *qnorm* function in R. N/A indicates that 95% confidence intervals could not be calculated at the given sampling point.

Sampling Point	Mean LT_{50} (°C)	Upper 95% CI (°C)	Lower 95% CI (°C)
Early Winter 2017	-13.2	-11.7	-14.7
Mid Winter 2017	-14.8	-12.6	-16.9
Late Summer 2017	-5.3	N/A	N/A
Fall 2017	-15.5	-11.5	-19.5
Early Winter 2018	-17.5	N/A	N/A
Mid Winter 2018	-15.3	-13.7	-16.8
Late Winter 2018	-14.2	-11.9	-16.5

3.1.3 Water Balance

On average, female *H. halys* survived desiccating conditions 14 % longer than males ($F_{1,96} = 24.51$, $p < 0.001$; Figure 3.7A). Moreover, *H. halys* was capable of surviving desiccating conditions longer in the winter compared to summer ($F_{9,96} = 3.50$, $p < 0.001$; Figure 3.7A), suggesting enhanced desiccation resistance in overwintering adults. On average, water content was higher in early-, mid-, and late-winter 2017/18 compared to other seasonal sampling points ($F_{9,96} = 2.89$, $p = 0.005$; Figure 3.7B), and greater in females compared to males ($F_{1,96} = 26.13$, $p < 0.001$; Figure 3.7B). By contrast, water content at death was greatest in mid-summer 2017 ($F_{9,96} = 2.57$, $p = 0.01$; Figure 3.7D), which does not coincide with peak water content. Interestingly, water content did not change during winter 2016/17 and winter 2017/18, as *H. halys* appeared to maintain their overall water balance (Figure 3.7B); this suggests that *H. halys* did not suffer significant desiccation stress while overwintering. Furthermore, water loss rate was reduced in the winter compared to summer ($F_{9,96} = 34.95$, $p < 0.001$), while males lost water at a slower rate than females ($F_{1,96} = 9.09$, $p < 0.004$; Figure 3.7C).

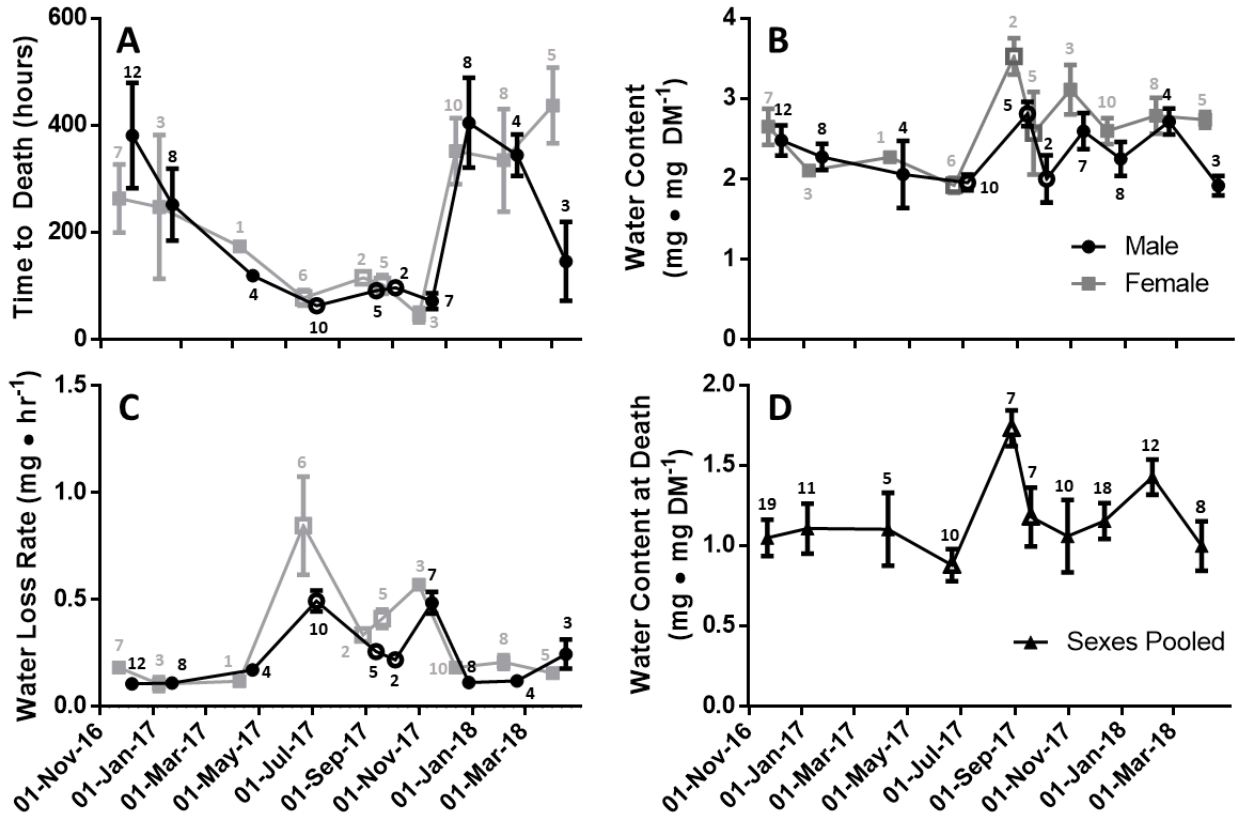

Figure 3.7 Seasonal variation in time to death (A), water content (B), water loss rate (C), and water content at death (D) in adult *Halyomorpha halys* (males and females pooled in D). Water balance of *H. halys* was determined gravimetrically using desiccation vials (Figure 2.3): adults were weighed prior to desiccation to determine fresh mass, after death to determine mass at death, and after drying in an oven at 60 °C (for a minimum of two days) to determine dry mass. Results are shown as mean (± SEM) of n = 1 - 10 adult male and female *H. halys* compared across sampling points using ANCOVA with dry mass as a covariate (numbers adjacent to each data point indicate sample size). Filled and open symbols indicate winter and summer sampling points, respectively.

3.1.4 Energy stores

Lipid content (mg · mg protein $^{-1}$) of adult *H. halys* was greater in the summer relative to winter (**(**$F_{9,116}$ = 2.62, $p < 0.001$), and greater overall in females compared to males ($F_{1,116}$ = 14.36, $p < 0.009$, Figure 3.8A). Female lipid content spiked in early summer and late summer 2017, before rapidly declining in fall 2017; a similar pattern was not seen in males (Figure 3.8A). Carbohydrate content (mg · mg protein $^{-1}$) in *H. halys* was also greater in summer relative to winter ($F_{9,116}$ = 6.38, $p < 0.001$), and greater overall in females compared to males ($F_{1,116}$ = 16.24, $p < 0.001$; Figure 3.8B). Moreover, female carbohydrate content spiked in early summer, mirroring the spike seen in female lipid content at the same time point (Figure 3.8B). Total energy content of *H. halys* was greater in females compared to males ($F_{1,117}$ = 14.48, $p < 0.001$; Figure 3.8C), but did not differ between seasons ($F_{9,117}$ = 1.86, $p = 0.06$). I found no significant difference in either lipid or carbohydrate content during each overwintering period (Figure 3.8A,B); even after 6-7 months of overwintering, energy content did not decrease relative to energy stores prior to overwintering.

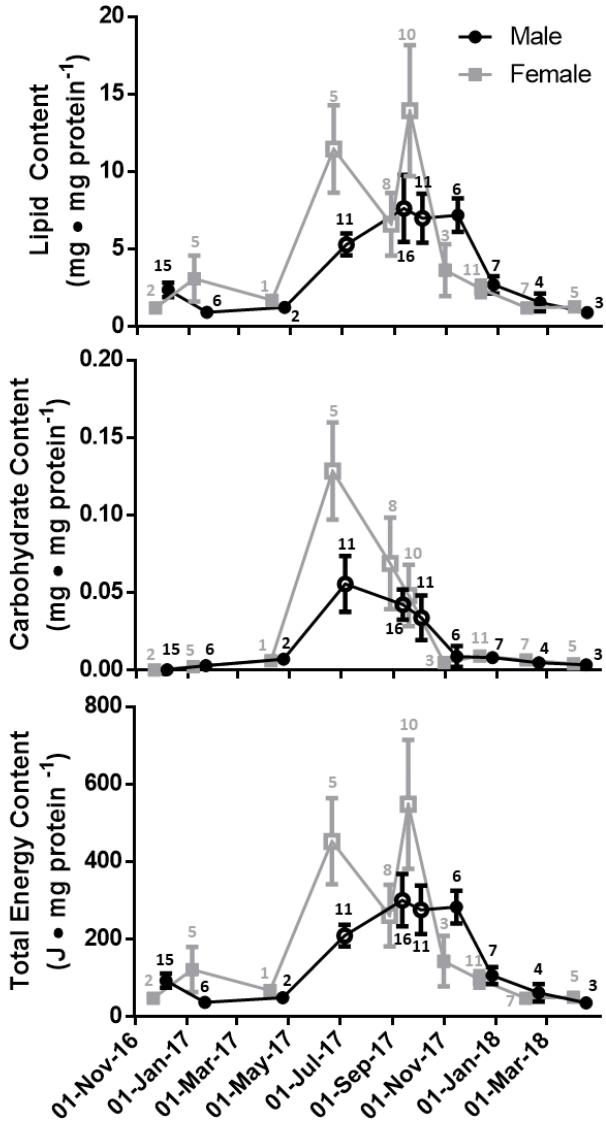

Figure 3.8 Seasonal variation in lipid (A), carbohydrate (B), and total energy content (C) of male and female *Halyomorpha halys*. Lipid and carbohydrate content were determined using modifications of the Folch method (Folch et al., 1956) and the anthrone method (Carroll et al., 1956; Williams et al., 2012a), respectively. Total energy content was calculated in joules assuming 39.3 J·mg lipid^{-1} and 17.6 J·mg carbohydrate^{-1} (Djawdan et al., 1998). Results are shown as mean (± SEM) of n = 1 - 16 adult male and female *H. halys* compared across sampling points using ANCOVA with soluble protein content as a covariate (numbers adjacent to each data point indicate sample size). Filled and open symbols indicate winter and summer sampling points, respectively.

3.2 Diapause

To assess the influence of diapause on stress tolerance, I reared separate laboratory colonies of *H. halys* under diapausing (short photoperiod; 8L:16D) and non-diapausing (normal photoperiod; 16L:8D) conditions at the same temperature (24 °C). In general, males weighed less than females in both diapausing and non-diapausing adults. Moreover, non-diapausing adults had greater fresh mass than diapausing adults, suggesting that they were larger in size ($F_{1,228} = 42.028$, $p < 0.001$). While I did not record any quantitative data, I noted that non-diapausing adults were much more active than diapausing adults when in rearing cages, and when handled. Diapausing *H. halys* tended to hide underneath paper towel and other substrate within rearing cages, and were noticeably less active (i.e. flying within cage) relative to non-diapausing *H. halys*. This is consistent with reports from Toyama et al. (2011), who found that diapausing *H. halys* seek dark refuge more often than non-diapausing *H. halys*, even when held at the same temperature.

I observed no instances of mating in diapausing rearing cages, nor did I find any egg masses during cage cleanings. In contrast, non-diapausing *H. halys* were observed mating within 2 - 3 weeks of adult emergence, and egg masses were retrieved from cages daily. I found that neither testis length, width, nor volume differed between treatments ($F_{1,36} = 2.82$, $p > 0.1$; $F_{1,36} = 0.09$, $p = 0.76$; $F_{1,36} = 0.50$, $p = 0.49$; Figure 3.9A,B). All diapausing females were categorized as unmated, and there was no evidence of mature (or immature) oocytes. I found minimal overlap in germarium and vitellarium width between treatments; in non-diapausing and diapausing females, germarium width ranged from 0.3 - 0.44 mm and from 0.24 - 0.46 mm, respectively, while vitellarium width ranged from 0.2 - 0.6 mm, and from 0.1 - 0.3 mm, respectively (Figure 3.10A,B). More importantly, I found that the germarium was wider than the vitellarium in all diapausing females (Figures 3.9C, 3.10A,B), indicating immature ovarioles. By contrast, mature oocytes were found in 60 % of non-diapausing females, while the vitellarium was wider than the germarium in 90% of the remaining females (Figures 3.9D, 3.10A,B). Overall, I measured more ovarioles in diapausing females relative to non-diapausing (Figure 3.10A,B), because mature oocytes were found in a majority of non-diapausing females, and I was unable to determine germarium and vitellarium width in these individuals.

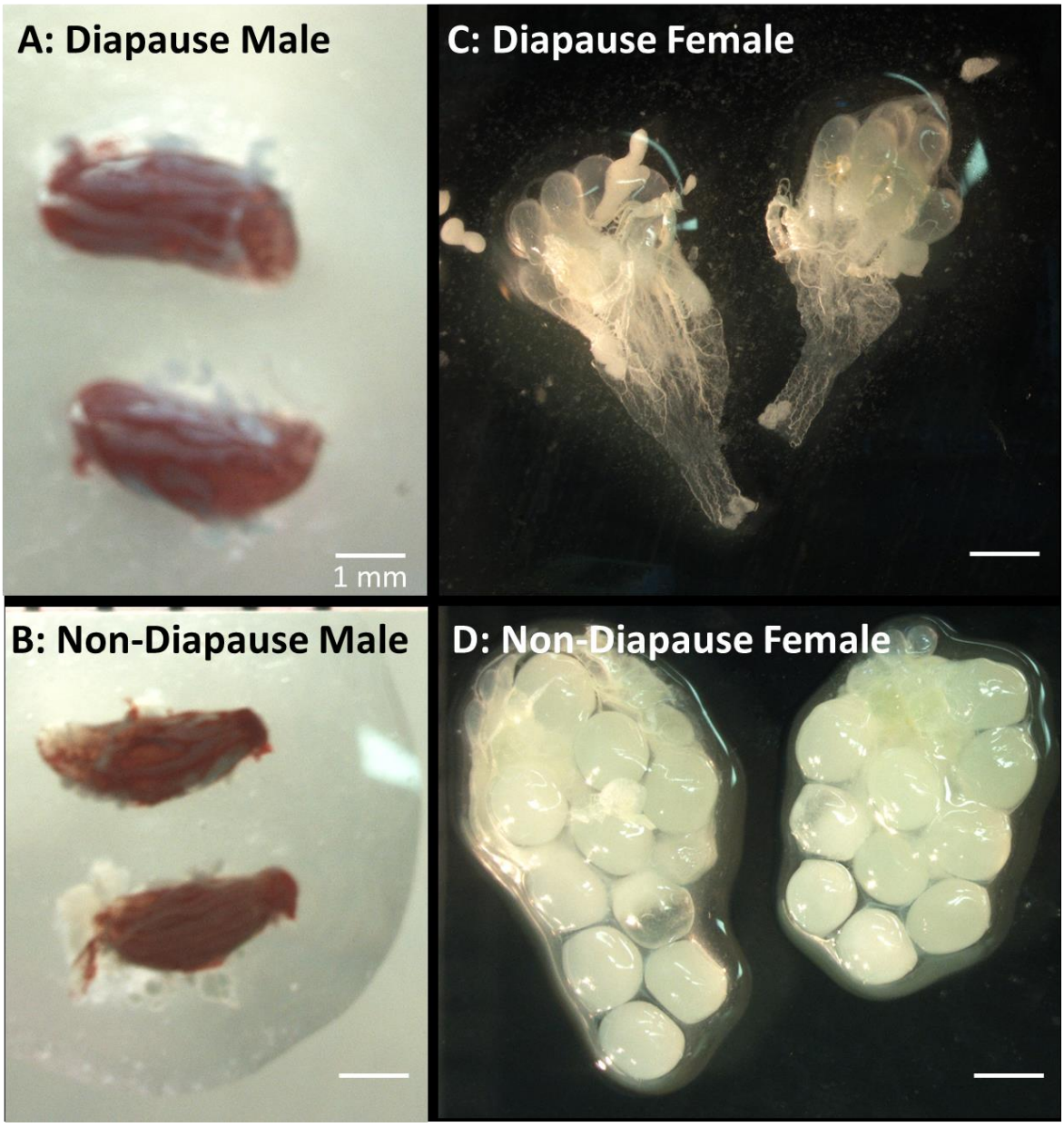

Figure 3.9 Testes removed from a diapausing male *Halyomorpha halys* (A) and a non-diapausing male (B), and ovaries removed from a diapausing female (C), and a non-diapausing female (D). Testes and ovaries were transferred to Ringer's solution on a microscope slide, after which I took pictures of each pair of organs using a camera (Nikon digital sight DS-Fil) installed on a stereomicroscope (Nikon SMZ 1500). All dissections were performed on adults 2 - 3 weeks after eclosion.

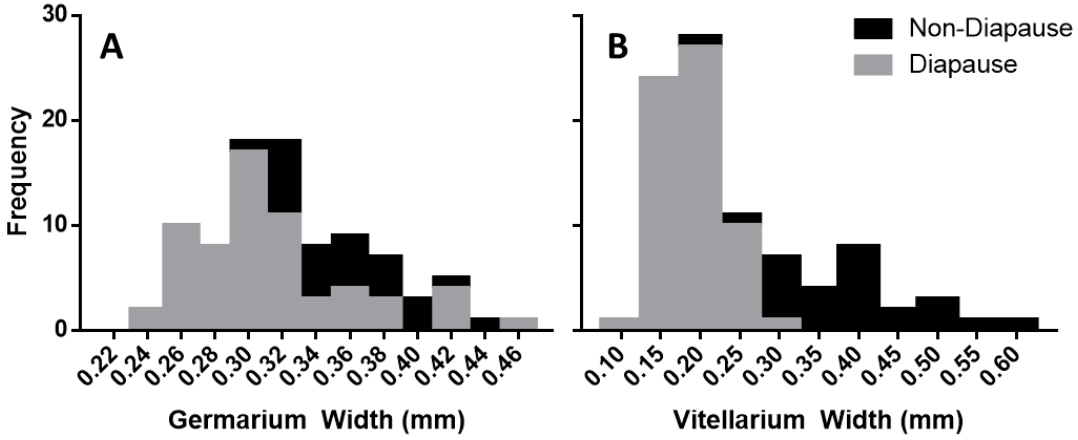

Figure 3.10 Germarium (A) and vitellarium (B) widths of laboratory-reared diapausing and non-diapausing adult female *Halyomorpha halys* (n = 10 individuals per rearing condition). Germarium and vitellarium width of diapausing and non-diapausing adult *H. halys* females were measured in ImageJ (ImageJ Developers) after being dissected and transferred to Ringer's solution, and photographed using a camera (Nikon digital sight DS-Fil) installed on a stereomicroscope (Nikon SMZ 1500).

3.2.1 Cold tolerance

Diapausing *H. halys* had lower SCPs than non-diapausing *H. halys* ($F_{1,55} = 5.1$, $p = 0.02$; Figure 3.11), while there was no effect of sex on the SCPs of either treatment group. Moreover, diapausing and non-diapausing SCP did not differ from overwintering adults at any time point, and were lower than SCPs recorded at summer sampling points ($F_{11,116} = 23.39$, $p < 0.001$). Lethal temperature (LT_{50}) of diapausing and non-diapausing *H. halys* did not differ ($t_{117} = 1.24$, $p = 0.19$; Figure 3.11; Table 3.2), nor did they differ from overwintering *H. halys* (late summer 2017 and early winter 2018 excluded). *Halyomorpha halys* from each rearing condition were unable to tolerate internal ice formation and were therefore not freeze-tolerant. More than 50 % of individuals which were exposed to temperatures slightly above the average SCP did not survive, suggesting that both diapausing and non-diapausing *H. halys* are chill-susceptible.

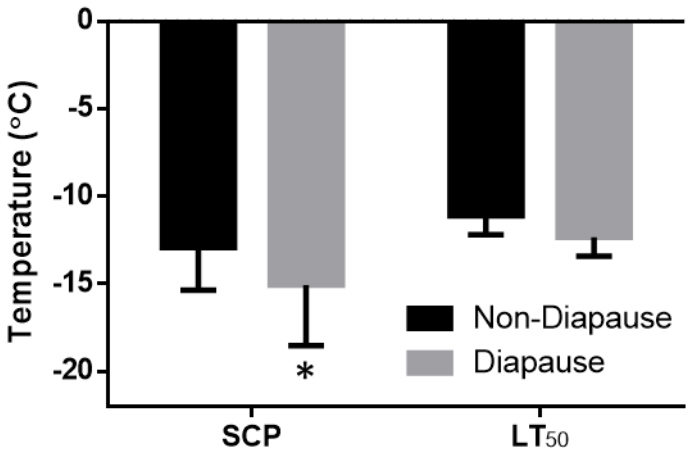

Figure 3.11 Supercooling point and LT_{50} of laboratory-reared diapausing and non-diapausing adult male and female *Halyomorpha halys.* Supercooling point was determined by cooling adults to -30 °C (in contact with a 36-AWG type-T thermocouple) and recording the lowest temperature prior to the exotherm. The temperature at which 50 % of adults were dead (LT_{50}) was calculated with a generalized linear model (with a binomial error distributions and logit function) after exposing groups of 4 - 5 adults to a temperature ranging from -5 °C to -20 °C for 1 h. Supercooling point results are shown as mean SCP (± SEM) of n = 26 - 33 specimens per treatment group. Asterisk indicates a significant difference between treatment resulting from ANCOVA with fresh mass as a covariate ($F_{1,53} = 5.69$, $p = 0.02$). LT_{50} results shown are mean LT_{50} ± 95 % CI of n = 25 *H. halys* per treatment group. 95 % confidence intervals were calculated using the *qnorm* function in R.

Table 3.2 Lethal temperatures (LT_{50}) of laboratory-reared diapausing and non-diapausing *Halyomorpha halys*. Groups of 4 - 5 adult *H. halys* were exposed to a single temperature ranging from -5 °C to -20 °C for 1 h. The temperature at which 50 % of adults were dead (LT_{50}) was calculated with a generalized linear model (with a binomial error distributions and logit function). 95 % confidence intervals (CI) were calculated using the *qnorm* function in R.

Treatment	Mean LT_{50} (°C)	Upper 95% CI	Lower 95% CI
Non-Diapause	-11.1	-10.0	-12.2
Diapause	-12.4	-11.3	-13.4

3.2.2 Water balance

Diapausing *H. halys* survived in desiccation chambers approximately five times longer than non-diapause individuals ($F_{1,47}$ = 42.93, $p < 0.001$; Figure 3.12A). Moreover, diapausing and non-diapausing *H. halys* did not differ in initial water content ($F_{1,47}$ =1.58, $p = 0.22$), whereas females had more absolute water compared to males overall ($F_{1,47}$ = 25.54, $p < 0.001$); however, there was no difference in water content between sexes proportional to body size (i.e. dry mass; Figure 3.12B). In non-diapausing *H. halys,* there was a sex × treatment interaction as water content was greater in females than males ($F_{1,47}$ = 7.94, $p = 0.007$). Diapausing *H. halys* had a reduced water loss rate compared to non-diapausing individuals ($F_{1,47}$ = 31.19, $p < 0.001$; Figure 3.12C), which is consistent with water loss rate in overwintering *H. halys;* however, there was no apparent effect of sex on water loss rate ($F_{1,47}$ = 1.01, $p = 0.32$). Additionally, I found that water content at death was lower in diapausing *H. halys* ($F_{1,47}$ = 15.05, $p < 0.001$; Figure 3.12D) and was lower in non-diapausing males compared to non-diapausing females as indicated by a sex × treatment interaction ($F_{1,47}$ = 25.98, $p < 0.001$).

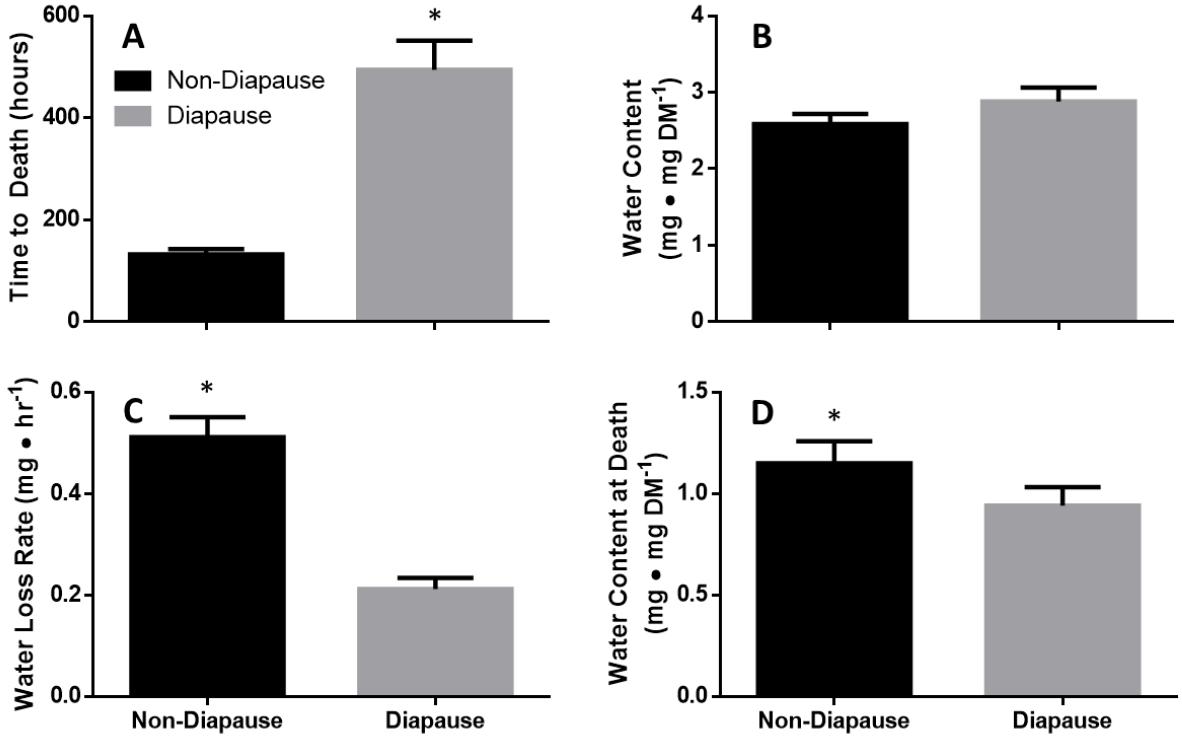

Figure 3.12 Desiccation time (A), initial water content (B), water loss rate (C) and water content at death (D) of laboratory-reared diapausing and non-diapausing adult *Halyomorpha halys* (males and females pooled in all figures). Water balance of *H. halys* was determined gravimetrically using desiccation vials (Figure 2.3); adults were weighed prior to dessication to determine fresh mass, after death to determine mass at death, and after drying in an oven at 60 °C (for a minimum of two days) to determine dry mass. Results shown are mean values (± SEM) of n = 25 - 27 adult *H. halys* per treatment group, determined using ANCOVA with dry mass as a covariate. Asterisks indicate a significant difference between treatment groups ($F_{1,47}$ = 42.93, $p < 0.001$ in A, $F_{1,47}$ = 31.19, $p < 0.001$ in C, and $F_{1,48}$ = 15.05, $p < 0.001$ in D).

3.2.3 Energy Stores

There was no difference in lipid content (mg · mg protein $^{-1}$) between laboratory reared diapausing and non-diapausing *H. halys* ($F_{1,32} = 0.18$, $p = 0.67$; Figure 3.13A), nor between males and females ($F_{1,32} = 0.000$, $p = 0.99$). Additionally, there was no difference in carbohydrate content (mg · mg protein $^{-1}$) between diapausing and non-diapausing *H. halys* ($F_{1,32} = 1.08$, $p = 0.31$; Figure 3.13B), nor between males and females ($F_{1,32} = 0.09$, $p = 0.77$). Moreover, total energy content did not differ between treatments ($F_{1,32} = 0.18$, $p=0.67$; Figure 3.13C), nor between sexes ($F_{1,32} = 0.00$, $p = 0.99$). When compared to field-collected *H. halys* from 2016/17 and 2017/18, laboratory-reared diapausing and non-diapausing *H. halys* had greater lipid stores than overwintering *H. halys* ($F_{11,154} = 6.31$, $p < 0.001$), while carbohydrate stores were only lower than *H. halys* sampled in early- -summer 2017, and greater than overwintering *H. halys* ($F_{11,154} = 7.44$, $p < 0.001$). In laboratory-reared diapausing and non-diapausing *H. halys*, lipids were the most abundant source of energy, which is consistent with overwintering and summer-collected adults.

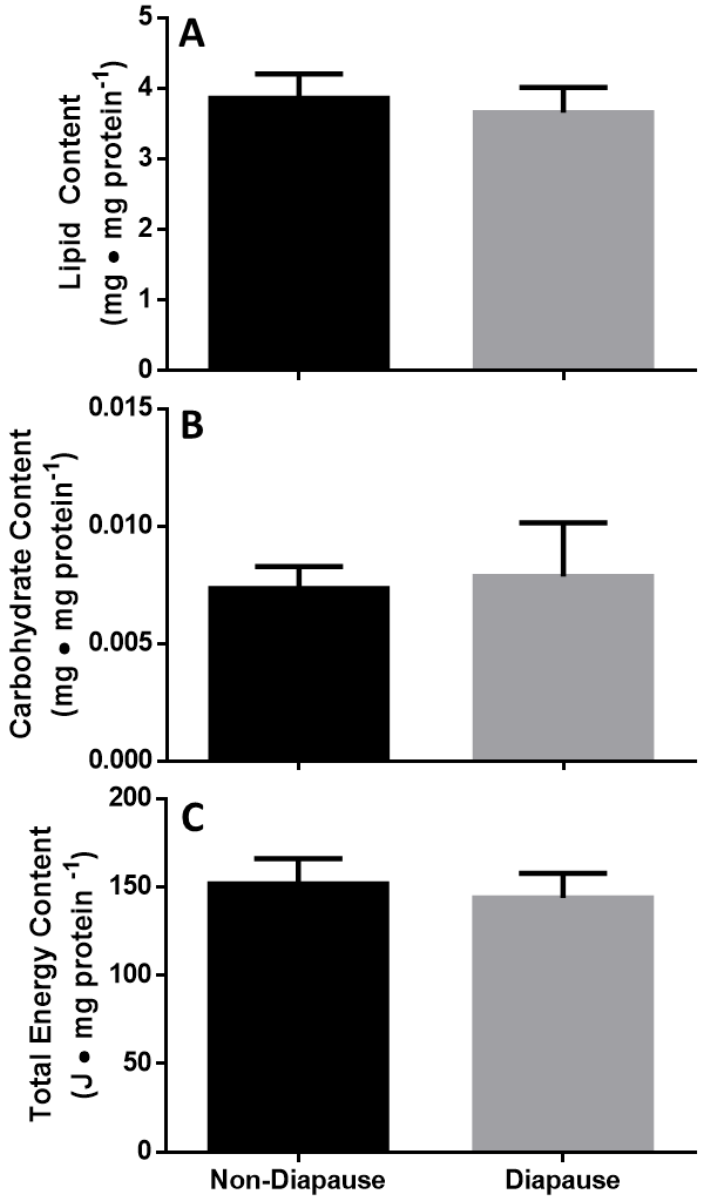

Figure 3.13 Lipid (A), carbohydrate (B), and total energy content (C) of laboratory-reared diapausing and non-diapausing adult *Halyomorpha halys* (males and females pooled). Lipid and carbohydrate content were determined using modifications of the Folch method (Folch et al., 1956) and the anthrone method (Carroll et al., 1956; Williams et al., 2012a), respectively. Total energy content was calculated in joules assuming 39.3 J·mg lipid^{-1} and 17.6 J·mg carbohydrate^{-1} (Djawdan et al., 1998). Results shown are mean (± SEM) of n = 18 - 20 *H. halys* per treatment group, as determined with ANCOVA with soluble protein as a covariate.

3.2.4 Metabolic rate

All adults showed continuous gas exchange (Figure 3.14A) at temperatures between 15 °C and 25 °C, whereas 50 % of non-diapausing *H. halys* showed discontinuous gas exchange (Figure 3.14B) at 10 °C, and 100 % of diapausing and non-diapausing adults showed discontinuous gas exchange at 5°C. Individuals at lower temperatures respired less than individuals at higher temperatures ($F_{4,76} = 98.52$, $p < 0.001$), males respired less overall than females ($F_{1,76} = 6.86$, $p = 0.01$), and diapause individuals respired less than non-diapause individuals at 5, 15, 20 and 25 °C, indicated by a temperature × treatment interaction ($F_{4,76} = 3.49$, $p < 0.02$; Figure 3.15). Additionally, at the 10 – 20°C temperature range I calculated Q_{10} values of 2.67 and 3.19 for diapausing and non-diapausing *H. halys*, respectively.

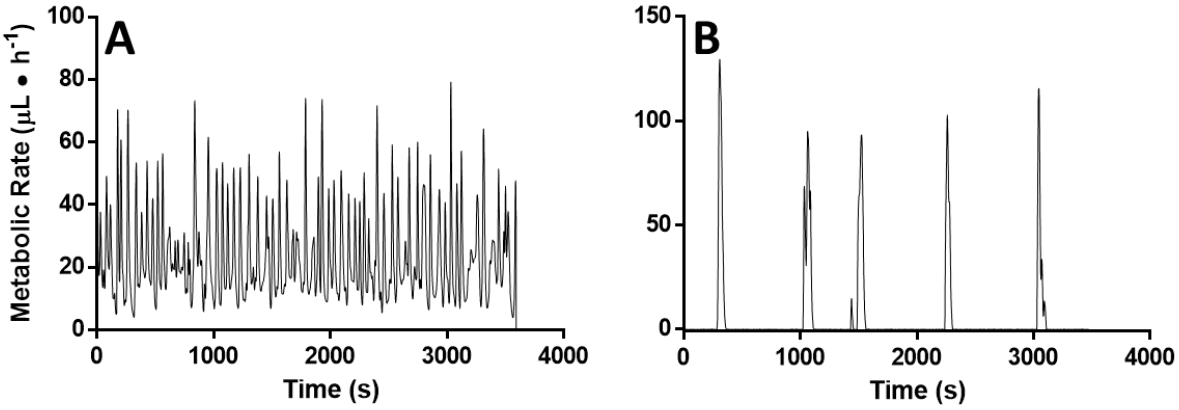

Figure 3.14 Recordings of metabolic rate from laboratory-reared adult *Halyomorpha halys*. Two distinct gas exchange patterns were observed in adult *H. halys*; continuous gas exchange in a 146.7 mg non-diapausing female at 25 °C (A), , and discontinuous gas exchange in a 168.6 mg diapausing female at 5 °C (B).

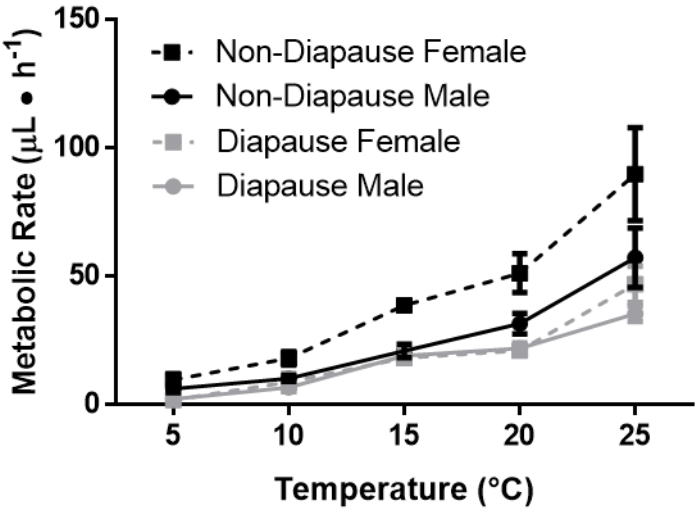

Figure 3.15 Metabolic rate of male and female laboratory-reared diapausing and non-diapausing *Halyomorpha halys* along a temperature gradient. CO_2 production of diapausing and non-diapausing adult *H. halys* was measured at 5, 10, 15, 20 and 25 °C following methods outlined by Williams et al. (2015a). Results shown are mean $\dot{V}CO_2$ (± SEM) of n = 2 - 7 adult male and female *H. halys* per treatment group at each temperature, and were determined using ANCOVA with fresh mass as a covariate. Dashed lines represent non-diapausing (grey) and diapausing (black) females.

Cuticular water loss was the largest source of water loss in both diapausing and non-diapausing *H. halys* and contributed to more than 85 % of total water loss in both treatment groups at each temperature. Diapausing *H. halys* had lower CWL rates than non-diapausing individuals ($F_{1,85}$ = 5.89, p = 0.02; Figure 3.16A), while CWL increased with temperature ($F_{4,85}$ = 3.16, p = 0.01). Diapausing *H. halys* had lower RWL rates compared to non-diapausing ($F_{1,87}$ = 28.15, p < 0.001; Figure 3.16B), while there was a temperature × treatment effect ($F_{1,87}$ =4.54, p < 0.003), indicating that RWL increases with temperature. Similar to CWL, there was no effect of sex on RWL rate. Additionally, RWL accounted for less than 15 % of overall water content at each temperature in both diapausing and non-diapausing *H. halys*, suggesting that CWL is more important for regulating water balance and enhancing desiccation resistance.

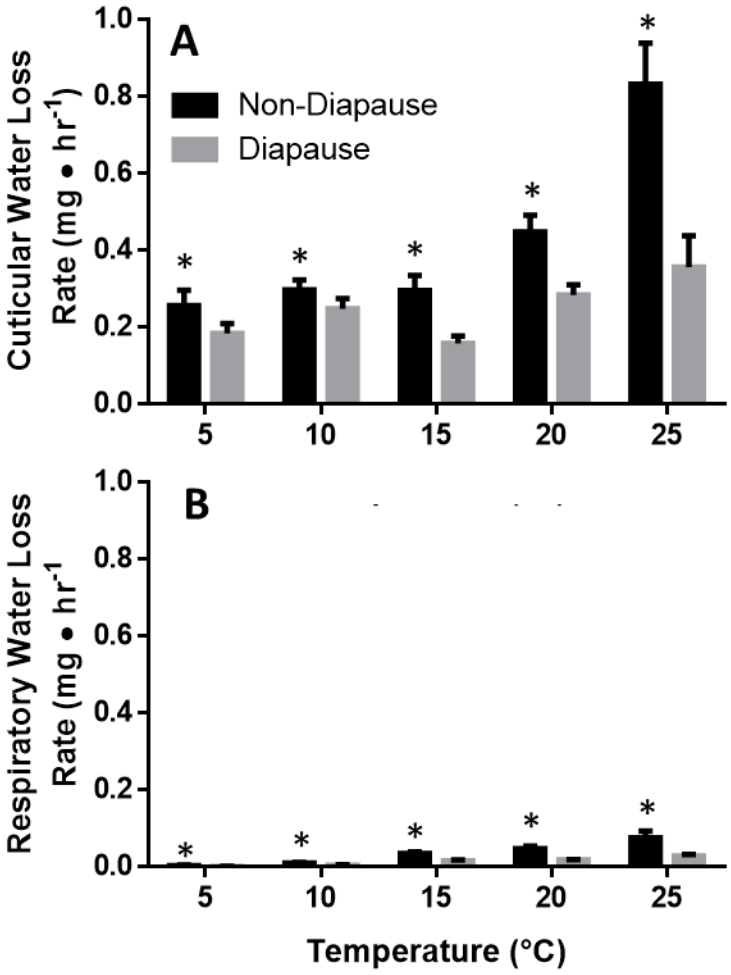

Figure 3.16 Cuticular water loss rate (A) and respiratory water loss rate (B) of laboratory-reared diapausing and non-diapausing adult male and female *Halyomorpha halys* across a temperature gradient. CO_2 and H_20 production of diapausing and non-diapausing adult *H. halys* were measured at 5, 10, 15, 20 and 25 °C following methods outlined by Williams et al. (2015a).Cuticular and respiratory water loss rates were calculated as the intercept of the regression of $\dot{V}H_2O$ against $\dot{V}CO_2$, and the difference between $\dot{V}H_2O$ and cuticular water loss rate, respectively (Gibbs and Johnson, 2004). Results shown are mean (± SEM) of n = 7 - 12 *H. halys* per treatment group at each temperature. Asterisks indicate a significant difference between treatment groups ($p < 0.05$) according to ANCOVA with fresh mass as a covariate.

4 Discussion

The main objectives of my project were to a) determine the relative importance of low temperatures, desiccation, and energy depletion in determining *H. halys* overwintering success, b) determine if *H. halys* stress tolerance is seasonally plastic, and c) determine the role of diapause in enhancing *H. halys* stress tolerance. Determining the extent of *H. halys* stress tolerance is important to develop a comprehensive understanding of *H. halys* biology and may prove beneficial in the development of novel pest management strategies.

4.1 Overwintering field mortality

I placed overwintering *H. halys* in one of two locations - an urban garden, which represented a "natural" overwintering site, and in a garage, which represented a "protected" overwintering site. To mitigate exposure to sub-zero temperatures, many insects overwinter in thermally buffered microhabitats, which minimize low temperature exposure (Danks, 1996). In natural settings for example, many Pentatomid species – including *H. halys* - aggregate underneath loose tree bark and leaf litter (Saulich and Musolin, 2012), which provide a degree of protection from environmental stressors. However, aggregations of overwintering *H. halys* in North America frequently occur in anthropogenic structures (Gariepy et al., 2014a), which may offer greater protection from environmental stressors. Thus, I only measured stress tolerance in *H. halys* overwintering in the garage, given their tendency to selectively overwinter indoors in North America (Inkley, 2012; Gariepy et al., 2014a), and given that almost all adults overwintered outdoors had died.

Mortality of overwintering *H. halys* was likely the result of chilling injury, desiccation, and/or energy depletion . Low temperatures limit insect performance and are influential in determining species' distribution. I found that *H. halys* were never exposed to temperatures near their SCP or LT_{50} when overwintering in a protected microhabitat; this provides evidence that *H. halys* mortality is not associated with freezing, and is more likely a result of chilling injury. However, chilling injury is dependent on both the degree of low temperature and the duration of exposure; given that the survival of *H. halys* overwintering outdoors decreased at a faster rate compared to those overwintering indoors, it is likely that adults overwintering outdoors are more likely to

accumulate lethal chilling injuries (e.g. loss of membrane ion balance, protein denaturation), and their survival is driven primarily by the degree of low temperature exposure. Additionally, *H. halys* overwintering outdoors were exposed to sub-zero temperatures for more than 50 days in both 2016/17 and 2017/18. By contrast, individuals housed indoors were only exposed to sub-zero temperatures at two acute (less than one hour) instances in each of 2016/17 and 2017/18 (Figure 3.1A, B). The degree of thermal buffering in protected overwintering microhabitats is therefore greater than natural sites. While the degree of low temperature exposure may dictate overwintering survival in a natural environment, the duration of low temperature exposure (e.g. less than 10 °C) may influence survival of *H. halys* overwintering indoors. Lowenstein and Walton (2018) found that acute, repeated cold exposure had no immediate or long-term impact on *H. halys* post-diapause survival, however acute effects of cold exposure may be less realistic than the effects of chronic long-term cold exposure experienced by overwintering *H. halys*. Lethal time can be determined following methods outlined by Sinclair et al. (2015), where adults are held at an ecologically-relevant temperature (e.g. 0 °C to -15 °C), and their survival assessed at various time intervals. Measurements of lethal time may prove beneficial in *H. halys* population management; for example, chilling shipping containers for a prolonged, predetermined time period - during or prior to shipment – could act as a control measure for hitch-hiking *H. halys*.

In addition to low temperatures and chilling injuries, desiccation likely influences *H. halys* overwintering success. When spending extended periods of time under desiccating conditions, an insect may use one of several strategies to prolong survival including; a) increase their water stores, b) increase their tolerance to water loss, and c) reduce their water loss rate (Gibbs and Johnson, 2004). In *H. halys*, water content did not change during either winter 2016/17 or 2017/18 (Figure 3.7B). Moreover, water content at death did not change across seasonal time points, indicating no changes in the lethal limits of *H. halys* water loss (Figure 3.7D). I found that overwintering *H. halys* actively reduce their WLR in the winter (Figure 3.7C), indicating a degree of desiccation resistance in overwintering adults. Thus, overwintering *H. halys* appear to maintain water balance through reduced water loss rates, prolonging their survival under desiccating conditions (Figure 3.7A). In an environment where extreme low temperatures are rarely experienced (e.g. indoors), strategies for combatting desiccation may be more important in ensuring overwintering success and post-winter emergence.

Energy depletion is likely an additional source of overwintering mortality in *H. halys*, as there was a lack of accessible food resources in overwintering microhabitats. I found that there was no change in either lipid or carbohydrate content through each overwintering period (Figure 3.8A, B), however, I did not quantify energy stores of deceased *H. halys.* If energy depletion was a limiting factor in *H. halys* overwintering success, I would expect that lipid and/or carbohydrate stores would be lower in a deceased *H. halys* relative to those which were alive at each sampling point. Moreover, insects that were found dead may have entered overwintering sites with less energy stores than those who survived, and simply consumed them at a faster rate. In the future, it would be useful to quantify energy stores of both live-sampled and dead-sampled overwintering *H. halys* to determine if there is a lethal limit of energy depletion , similar to that of water content (i.e. water content at death).

4.2 Overwintering and diapause stress tolerance

4.2.1 Cold tolerance

I found that both male and female *H. halys* adults are incapable of surviving at temperatures below or equal to their SCP, which ranged from -5.3 °C in the summer to -17.5 °C in the winter (Figures 3.3, 3.4). *Halyomorpha halys* are therefore chill-susceptible, and their mortality at low temperatures is the result of chilling injuries rather than freezing injuries. I found that the LT_{50} (temperature at which 50 % of individuals die after one hour of cold exposure) of both male and female *H. halys* is seasonally plastic, and ranges from -5.3 °C in the summer to -17.5 °C in the winter (Figure 3.5; Table 3.1). My findings on *H. halys* cold tolerance strategy are consistent with Cira et al. (2016), who found that *H. halys* are chill-susceptible, and depress their SCP to approximately -17 °C in the winter. However, Cira et al. (2016) failed to measure LT_{50} despite finding that *H. halys* was chill-susceptible; as such, it is not appropriate to draw conclusions about *H. halys* cold hardiness based on their study. Several other stink bug species, including the harlequin bug *Murgantia histrionica* (Hemiptera: Pentatomidae) and *N. viridula* are also chill-susceptible as overwintering adults (Elsey, 1993; DiMeglio et al., 2016). This appears to be common within Hemiptera peach-potato aphids, *Myzus persicae* (Hemiptera: Aphididae), which overwinter as obligate parthenogens (i.e. active nymphs and adults) are chill-susceptible, but

enhance their cold hardiness (i.e. lower their LT_{50}) following acclimation at low temperatures (Clough et al., 1990; Bale, 1996; Vorburger, 2004).

In *H. halys,* acclimitization to low temperatures enhances cold tolerance through the depression of SCP and LT_{50} in the winter. Acclimatization can enhance cold hardiness through a variety of mechanisms, including the accumulation of hemolymph cryoprotectants. I found that hemolymph osmolality was greater at two time points in winter 2017/18 compared to summer 2018 (Figure 3.6), however the degree of osmolyte accumulation was not significant enough to conclude that hemolymph cryoprotectants are solely responsible for SCP and/or LT_{50} suppression. By contrast, in the stink bug *Graphosoma lineatum* (Hemiptera: Pentatomidae) an accumulation of hemolymph cryoprotectants through acclimation is responsible for SCP depression and the subsequent enhancing of cold hardiness (Šlachta et al., 2002). Hemolymph cryoprotectants can be characterized following methods like those of Crosthwaite et al. (2011) by processing samples through gas chromatography coupled with mass spectrometry to identify which molecular compounds are present, if any, in overwintering *H. halys.* Alternatively, acclimatization may minimize the risk of chilling injury in *H. halys,* thus enabling prolonged survival at low temperatures. In the spring field cricket, *Gryllus veletis* (Orthoptera: Gryllidae), an increase in Na^+ content in lab-acclimated crickets may provide greater tissue resistance to ion leaking during cold exposure, delaying chill-coma onset and any subsequent chilling injuries (Des Marteaux and Sinclair, 2016). By contrast, acclimation of *D. melanogaster* at 15 °C reorganizes the overall composition of membrane phospholipids, resulting in increased membrane stability and a 1.5 °C decrease in LT_{50} (Overgaard et al., 2008). Moreover, in *G. pennsylvanicus,* cold acclimation increases F-actin density and promotes actin stability (Des Marteaux et al. 2017, 2018), which appears to be important for enhancing cytoskeletal stability and improving survival after cold exposure (Kim et al., 2006; Des Marteaux et al., 2017). It remains unclear which mechanism - if any of the aforementioned - contribute to enhanced cold tolerance of overwintering *H. halys;* however, such information would be useful and will inform as to the degree of chilling *H. halys* can sustain.

Diapausing and non-diapausing adults of *H. halys* are both chill-susceptible, which is consistent with overwintering adults. Average SCP was lower in diapausing *H. halys* compared to non-diapausing, however, LT_{50} did not differ between treatments (Figure 3.11, Table 3.2). In *G.*

lineatum, cold tolerance and diapause appear to be linked; diapausing adults depress their supercooling point from -7 °C in the summer to -18 °C in the winter, which coincides with an increase in hemolymph trehalose levels (Šlachta et al., 2002). Cold tolerance and diapause are independent phenomena in other Pentatomid species, however, as diapausing and non-diapausing adult *N. viridula* show no difference in SCP or LT_{50} (Elsey, 1993). In *N. viridula*, diapause may therefore act as an adaption for regulating life cycle rather than a mechanism for surviving low temperatures. This appears to be the case in *H. halys*, as neither the SCP or LT_{50} of lab-reared diapausing and non-diapausing *H. halys* differed from those of overwintering adults in 2016/17 or 2017/18.

Plasticity of *H. halys* cold hardiness in the field is therefore likely induced through acclimatization, rather than solely as a by-product of the diapause program (Denlinger, 1991; Teets and Denlinger, 2013). During the winter, insects housed indoors and outdoors were both exposed to low temperatures (e.g. >10 °C) prior to assessing their cold tolerance; it is therefore likely that *H. halys* were acclimatized to low temperatures at the time of sampling (Teets and Denlinger, 2013). Summer-collected *H. halys* were not exposed to low temperatures prior to assessment, which likely explains their lack of cold hardiness. Cold-hardiness is also present in the non-diapausing stage of insects; for example, in the flesh fly *Sarcophaga bullata* (Diptera: Sarcophagidae) non-diapausing pupae are less cold tolerant relative to their diapausing counterpart yet can achieve limited cold hardiness through rearing at low temperatures (Denlinger, 1972). The enhancement of cold hardiness in diapausing and non-diapausing pupae is the result of a shared mechanism; in both states, acclimation in the lab (i.e. rearing at low temperatures) is driven by increased glycerol concentrations (Denlinger, 1991). Similar mechanisms in *H. halys* are plausible and may explain why the SCP and LT_{50} of non-diapausing and overwintering *H. halys* do not differ.

4.2.2 Water Balance

In the field, I found differences in *H. halys* water content based on sex and season (Figure 3.7B). More importantly, I found no significant reduction of water content in *H. halys* during winter 2016/17 or 2017/18 (Figure 3.7B), indicating that they did not suffer from extreme desiccation. Given that water content at death did not change seasonally (Figure 3.7D), it is evident that *H.*

halys can maintain water balance throughout the year and are therefore desiccation tolerant. Additionally, water content of diapausing and non-diapausing *H. halys* did not differ between treatment groups (Figure 3.12B), providing further support of desiccation tolerance.

I found that *H. halys* water loss rate was reduced in winter 2016/17 and 2017/18 compared to the summer (Figure 3.7C); thus, it appears that *H. halys* were actively reducing their water loss rate as a strategy for resisting desiccation while overwintering. This is common in overwintering insects, as sub-zero temperatures intensify desiccation stress (Danks, 2000). Under lab conditions (i.e. approximately 0% RH at room temperature), overwintering females lost water at a rate of approximately 0.2 $mg \cdot hr^{-1}$, equating to 4.0 mg daily, while males lost water at that rate of 0.15 $mg \cdot hr^{-1}$, equating to 3.0 mg daily. Under field conditions, however, RH was >50 % in protected overwintering sites in both 2016/17 and 2017/18, and temperature rarely surpassed 10 °C; it is therefore likely that adults lost water at a slower rate in the field and may potentially survive longer under desiccation. Diapausing *H. halys* had a lower overall water loss rate than non-diapausing adults (Figure 3.12C), which is consistent with overwintering *H. halys*; this suggests that diapausing *H. halys* are also resistant to desiccation, which enables the maintenance of water balance. Reduction of water loss rate is common in diapausing insects, regardless of life stage. Diapausing pupae of the corn earworm *Helicoverpa zea* (Lepidoptera: Noctuidae), for example, suppress their water loss rate nearly two-fold compared to non-diapausing pupae **(**Benoit et al., 2015), while diapausing adults of the northern house mosquito *Culex pipiens* (Diptera: Culicidae), can reduce water loss by nearly 10% (Benoit and Denlinger, 2007). Given that cuticular water loss is the greatest source of water loss in *H. halys* (> 85 % of total water loss; Figure 3.16A), it is likely that a reduction in water loss rate is associated with a reduction of cuticular water loss. Cuticular water loss is often regulated through physiological modifications which reduce cuticular permeability; this could include increasing the overall quantity of cuticular lipids as in the diapausing pupae of *H. zea* (Hadley, 1994; Benoit et al., 2015), or through the lengthening of cuticular hydrocarbon chain length as in *D. melanogaster* (Bazinet et al., 2010). One of these strategies may explain the active reduction of water loss rate in overwintering *H. halys* and should be further investigated.

In many temperate species, the mechanisms underlying desiccation tolerance and cold tolerance overlap (Danks, 2000). The depression of an insect's SCP is often promoted through the

accumulation of hemolymph cryoprotectants, (Ring and Danks, 1994); as a result, insects that tend to be more cold-hardy also appear to be more desiccation tolerant (Sinclair et al., 2013). Reduced water content promotes an increase in solute concentration, which subsequently enhances supercooling and limits the amount of water free for ice-nucleation (Ring and Danks, 1994). This contradicts what is seen in *H. halys*, as SCP and LT_{50} are depressed in the winter (Figures 3.3, 3.5) independently of changes in water content (Figure 3.7B). Rather, only a reduction of WLR is seen (Figure 3.7C), which may simply serve as a mechanism for conserving water and enhancing desiccation resistance rather than desiccation tolerance or cold-tolerance. This may prove advantageous in overwintering environments were desiccation stress threatens survival to a greater degree than low temperature stress, as is the case with *H. halys* which overwinter in thermally buffered microhabitats.

4.2.3 Energetics

Both lipid and carbohydrate stores were lower during the fall and winter compared to summer in adult *H. halys* (Figure 3.8A,B) . This is consistent with Funayama (2012), who found that *H. halys* had a lower mass after emerging from overwintering sites relative to their pre-overwintering mass; in this study, lower mass was attributed to an individual's nutritional state. Additionally, *H. halys* had greater stores of lipid compared to carbohydrates (Figure 3.8A,B), suggesting that lipids are the primary energy source of *H. halys*. This is common in insects as lipids - triacylglycerols in particular - provide the greatest source of energy (Hahn and Denlinger, 2011). An increase in lipid and carbohydrate stores during the summer is not surprising, as *H. halys* had greater access to food sources, while overwintering individuals were not feeding once in their respective microhabitats. Prior to overwintering, many insects accumulate energy stores to fuel post-overwintering emergence, development and reproduction (Tauber et al., 1986); this was not the case in *H. halys*, however, as both carbohydrate and lipid stores of males and females declined significantly in the fall, prior to overwintering (Williams et al., 2012a; Figures 3.8A,B). Given that energy consumption is dictated in part by metabolic rate, I hypothesize that *H. halys* energy consumption between late summer and fall was driven by exposure to high temperatures while preparing for overwintering (Williams et al., 2012b).

I found no difference in either lipid or carbohydrate content across winter sampling points (Figure 3.8A,B); *H. halys* were therefore not consuming energy stores at a significant rate while overwintering. This is consistent with other diapausing stink bugs, including *Biprorulus bibax* (Hemiptera: Pentatomidae), which show no significant decline of lipid reserves through winter (James, 1990). Energy conservation is an important factor in dictating overwintering success, as insects may use remaining stores to emerge from dormancy and fuel spring reproduction (Hahn and Denlinger, 2011). Energy expenditure increases with temperature (Williams et al., 2012b) and metabolic rate is disproportionately higher as mean temperature increases; energy stores are therefore consumed at a faster rate relative to the amount of energy conserved at lower temperatures (Williams et al., 2012b; Sinclair, 2015). Warm spells are thus problematic for *H. halys,* and likely explain the reduction in energy stores seen during the shoulder seasons (i.e. fall and spring). Diapause and associated reductions in metabolic rate may prove advantageous in overwintering *H. halys* and may provide a mechanism for reducing energy consumption while overwintering.

I found that in both diapausing and non-diapausing *H. halys,* metabolic rate increased as a function of temperature; however, diapausing *H. halys* had reduced metabolic rates at 5, 15, 20 and 25 °C compared to non-diapausing adults, suggesting metabolic suppression in diapause (Figure 3.15). Metabolic suppression is a key characteristic of the diapause program and is seen in many other insects including the European spruce bark beetle *Ips typographus* (Coleoptera: Curculionidae), where overwintering adults have a reduced metabolic rate compared to those which have emerged (Schebeck et al., 2017). Given that Q_{10} did not differ greatly between diapausing ($Q_{10} = 2.67$) and non-diapausing ($Q_{10} = 3.19$) *H. halys.,* it appears that adults reared under each treatment are equally thermally sensitive; despite this, diapausing *H. halys* are still capable of metabolic suppression, indicating that metabolic suppression is likely temperature-independent. Temperature-independent metabolic suppression is a key feature of diapause and reduces overall energy expenditure (Tauber et al., 1986; Hahn and Denlinger, 2011; Sinclair, 2015). Moreover, metabolic suppression in *H. halys* is consistent with my field results and provides an explanation for limited lipid and carbohydrate consumption seen in overwintering adults (Figures 3.8A,B). In addition, microhabitat selection is likely important for regulating energy expenditure in overwintering insects (Pauli et al., 2013; Sinclair, 2015), including *H. halys*. This phenomenon is seen in the Arctic woollybear caterpillar, *Gynaephora groenlandica*

(Lepidoptera: Lymantriidae), , where caterpillars overwintering in hibernacula underneath rocks experience a higher degree of thermal stability than those which overwinter in grasses and leaf litter, likely contributing to increased energetic savings (Bennett et al., 2003). . Because *H. halys* overwinter indoors, they are buffered from significant temperature fluctuations (e.g. >15 °C in a 24 h period; Figure 3.1A,B) and any subsequent exponential increases in metabolic rate which may be experienced in natural (outdoor) overwintering sites, and therefore suffer minimal energy depletion.

I found that female *H. halys* had greater lipid and carbohydrate stores in early summer, and greater lipid stores in late summer compared to males (Figure 3.8A,B). Females often have greater energy stores than males; from an evolutionary perspective, greater summer energy stores are advantageous for reproduction. In the cabbage beetle *Colaphellus bowringi* (Coleoptera: Chrysomelidae), for example, reproductive females store greater amounts of lipid and carbohydrates post-feeding compared to their pre-diapause counterparts (Tan et al., 2016). In Swiss *H. halys* populations, peak oviposition occurs in mid-late June (Haye et al., 2014); if this is true in Ontario populations, then peak oviposition coincides with an increase in female lipid and carbohydrate content (Figure 3.8A,B), which could be a response to the increased energetic demands of oviposition and egg production. Moreover, mature eggs within a female's ovaries can account for approximately 61 % of their overall lipid content and 20 % of their carbohydrate levels (Skillman and Lee, 2017), which may further explain the noted increases in lipid and carbohydrate content. Under natural conditions, development from egg to adult may take anywhere from 60 to 130 days (Haye et al., 2014); this second generation of new adults coincides with another spike in female lipid content in late summer (Figure 3.8A), which may indicate that females are reproductively active prior to overwintering. This suggests evidence of multivoltinism and a partial second generation of adults in southwestern Ontario, which was previously predicted by Gariepy et al. (2014a).

Neither lipid or carbohydrate content differed between diapausing and non-diapausing *H. halys* (Figure 3.13A,B). Additionally, both diapausing and non-diapausing *H. halys* had greater lipid stores than carbohydrates; this suggests that lipids were the primary source of energy in both treatments and is consistent with field results. Both diapausing and non-diapausing colonies were reared on identical diets and under the same thermal regime; as a result, energy stores of adults

from both treatments closely mirrored those of summer-collected *H. halys* rather than overwintering *H. halys.* It appears that under optimal conditions and with food available, *H. halys* will continue to feed regardless of reproductive diapause initiation. This is consistent with my observations of diapausing *H. halys,* which I observed feeding at various times throughout development, including as adults. Feeding is not entirely uncharacteristic of diapausing insects, as diapausing adults of the black blowfly *Phormia regina* (Diptera: Calliphoridae) are active and feed during diapause, albeit at a rate 80 % lower than their non-diapausing counterparts (Stoffolano, 1975). Moreover, exposure to low temperatures in overwintering *H. halys* may dictate feeding behaviour and subsequent energy depletion ; if metabolic rate is reduced at low temperatures (Hahn and Denlinger, 2011), less energy may be needed to fuel overwintering survival, and there is no need to restore energy stores.

Adult *H. halys* showed two distinct gas exchange patterns; at 15 °C and above, all individuals had continuous gas exchange (figure 3.14A). At 10 °C however, 50 % of non-diapausing individuals showed discontinuous gas exchange (DGC; Figure 3.14B) - with the remaining showing continuous - while at 5 °C all diapausing and non-diapausing individuals showed DGC. Shifts in gas exchange patterns are well documented in insects and believed to aid in tolerating stress. The hygric hypothesis of DGC suggests that discontinuous gas exchange is a mechanism to minimize respiratory water loss (Gibbs and Johnson, 2004), however I (and many other studies) have found that respiratory water loss accounts for less than 15 % of overall water loss, challenging the importance of DGC for water conservation (Gibbs and Johnson, 2004; Schilman et al., 2005; Chown et al., 2006). Alternatively, reduction of energy expenditure appears to be a common feature of species showing DGC (Matthews and White, 2010). I hypothesize that the onset of DGC and subsequent reduction of energy expenditure is a temperature mediated process in *H. halys*; as temperature decreases, *H. halys* may shift to DGC as a strategy to limit energy depletion while overwintering.

4.3 Ecological relevance of *H. halys* overwintering biology

I found that *H. halys* is capable of overwintering indoors in southern Ontario, but not outdoors. While they are chill-susceptible, overwintering *H. halys* avoid low temperature exposure by aggregating in thermally buffered microhabitats, and their overwintering success is therefore not

limited by low temperatures. Additionally, *H. halys* are desiccation resistant, evidenced by an active reduction of their WLR during extended periods of desiccation (Figure 3.7C). Lastly, *H. halys* suffer minimal energy depletion while overwintering; while there is a degree of seasonal plasticity to *H. halys* energy reserves, at no point do they suffer from significant lipid or carbohydrate depletion through the overwintering period (Figure 3.8A,B). The mitigation of energy depletion is very likely the result of active reduction of metabolic rate at low temperatures, as evidenced in diapausing *H. halys* (Figure 3.15). Moreover, overwintering in thermally buffered microhabitats reduces exposure to fluctuating temperatures (i.e. diurnal) and high temperatures, both of which drive energy consumption. Thus, it is likely that energy depletion is not a limiting factor in dictating *H. halys* overwintering mortality.

My results suggest that *H. halys* in southern Ontario are tolerant of all three stressors – low temperatures, desiccation, and energy depletion – when experienced individually under realistic overwintering conditions. When exposed to a single stressor during each experiment, I found that no sole stressor was a limiting factor in determining *H. halys* overwintering success. However, exposure to two or more stressors simultaneously is more probable in nature (Kaunisto et al., 2016); therefore, I predict that exposure to a combination of stressors is responsible for determining *H. halys* survival, and that the sublethal effects of multiple stressors may interact in a manner that induces mortality (under realistic overwintering conditions). Thus, investigating the interactions between stressors is important for developing a comprehensive overview of *H. halys* stress tolerance. This can be easily performed in lab by exposing *H. halys* to stressors simultaneously and assessing mortality afterwards. For example, to determine if there is an interaction between desiccation and low temperature exposure (i.e. is stress tolerance enhanced/worsened in anyway), *H. halys* could be desiccated for a given period (e.g. two weeks), and their cold tolerance measured immediately after. This could then be repeated with the inclusion of an additional stressor or in a different order. Completing such experiments would provide a greater understanding of *H. halys* stress tolerance under realistic overwintering conditions and may inform alternative pest management tactics.

Furthermore, I propose that similar assessments of stress tolerance should be performed on potential biological control agents of *H. halys* prior to their introduction to temperate North America. Several species of *Trissolcus* (Hymenoptera: Platygastridae), a genus of parasitioid

wasps, have been evaluated as potential biological control agents of *H. halys;* of those, cold tolerance has only been measured in two species, *T. japonicus*, which has an established population within the United States, and *T. cultratus* (Santacruz et al., 2017). Females of *Trissolcus* spp. parasitize egg masses of *H. halys.* While cold tolerance represents an important physiological trait, it does not encompass the entirety of an insect's overwintering constraints. To date, little information exists detailing the overall overwintering biology of *Trissolcus* spp.; it is unknown where they overwinter (Santacruz et al., 2017), and whether diapause is initiated prior to overwintering. I suggest that future studies further characterize the overwintering biology of *Trissolcus* spp. by determining; a) the degree to which *T. japonicus* and *T. cultratus* suffer from desiccation, b) the degree to which they suffer from energy depletion, and c) the mechanisms underlying their cold tolerance. This information will greatly inform the efficacy of a pest management strategy focused on these biological control agents by identifying which species is capable of overwintering successfully (if either) and should be released for potential management purposes.

4.4 Conclusions

This study is the first to examine multiple stressors which influence the overwintering success of *H. halys.* By overwintering in thermally buffered microhabitats, low winter temperatures in Ontario do not set boundaries for overwintering success in *H. halys.* Although acclimatization increased cold-hardiness in overwintering *H. halys*, adults do not experience low temperatures and are not at risk of extreme chilling. Rather, *H. halys* have developed strategies to tolerate both desiccation stress and energy depletion while overwintering. Adult *H. halys* maintain water balance through a reduction of water loss rate, promoting increased survival time under desiccating conditions. Additionally, *H. halys* conserve energy stores through winter, likely a result of metabolic suppression. Both strategies are linked to the diapause program in *H. halys*, which ultimately enhances their overwintering capabilities. Thus, *H. halys* are well-equipped to successfully overwinter indoors in Ontario and will continue to persist barring any significant changes in overwintering conditions. Given their inability to survive overwintering conditions in natural environments in Ontario, I suggest developing means of controlling *H. halys* from entering anthropogenic structures (i.e. physical or chemical barriers) and forcing them to overwinter outdoors.

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